Agriculture and Agri-Food Canada | Agriculture et Agroalimentaire Canada

ONTARIO RESEARCH ENHANCEMENT PROGRAM (O R E P)

Final Report

FUSARIUM RESISTANCE VIA BIOTECHNOLOGY

REPORT No. OREP - 1999/03

LEAD INVESTIGATOR
Jas Singh

Canada

103-02919/

ONTARIO RESEARCH ENHANCEMENT PROGRAM (O R E P)

Final Report

FUSARIUM RESISTANCE VIA BIOTECHNOLOGY

REPORT No. OREP-1999/03

August 2001

Project Leader
Dr. Jas Singh

Eastern Cereal and Oilseed Research Centre
Agriculture and Agri-Food Canada,
Central Experimental Farm
Ottawa, Ontario, K1A 0C6
Tel: 613-759-1662 Fax: 613-759-1701
Email: singhja@em.agr.ca

Collaborators:
Dr. Linda Harris
Dr. Steve Gleddie
Dr. Thérèse Ouellet
Dr. John Simmonds

Eastern Cereal and Oilseed Research Centre
Agriculture and Agri-Food Canada,
Central Experimental Farm
Ottawa, Ontario, K1A 0C6

FORWARD

The Ontario Research Enhancement Program (OREP) is a $4-million two-year federal research initiative that is administered by the Research Branch of Agriculture and Agri-Food Canada (AAFC), with input from the agriculture and agri-food sector, universities and the province. Research focuses on two areas identified by the sector as:

1. Priorities responding to consumer demand for higher quality products; and
2. Ensuring crop-production management systems are environmentally sustainable.

Biotechnology is one of the areas to be explored. Ontario's agricultural production is based primarily on growing diversified crops using intensive production practices, but long-term viability is linked to the development of sustainable crop production management systems.

There are also opportunities to enhance the economic contribution of Ontario's agriculture and agri-food sector by adding value to the diversified primary commodities produced in the province. The Program is expected to be of particular interest to the corn, soybean, greenhouse, fruit and vegetable sectors and the emphasis will be on projects focussing on food quality improvement and sustainable crop production management such as:

1. The development of value-added animal and crop food products and ingredients that meet domestic and export market demands, with a particular emphasis on quality issues such as processing, packaging, shelf life, new product and ingredient development, high-value alternative uses, probiotics and functional foods;
2. Biotechnologies to reduce environmental pressures related to crop protection practices; and,
3. Integrated crop protection and production practices for both field and greenhouse crops.

Program Manager:
Dr. Bruce T. Bowman
Southern Crop Protection and Food Research Centre (SCPFRC),
Agriculture & Agri-Food Canada
1391 Sandford St., London, ON N5V 4T3
Tel: (519) 457-1470 x239 Fax: (519) 457-3997
E-Mail: bowmanb@em.agr.ca
Program URL: http://res2.agr.ca/london/pmrc/english/orep/orephome.html

EXECUTIVE SUMMARY

Disease caused by *Fusarium graminearum* (gibberella or corn ear rot) is the single most serious threat to the Ontario corn crop. Mycotoxins produced on the grain are a major concern to livestock producers and the milling industry, and they can have a devastating effect on the value of the crop. Major epidemics occurred in 1982 and 1996 and losses in cash receipts to Ontario corn growers were estimated to be tens of millions of dollars in each of those years. Thus, we are looking at biotechnology strategies for identifying and developing novel *Fusarium* resistance in maize.

The mycotoxin DON enhances the spread of *Fusarium* in the plant. We have incorporated into corn a modified rice ribosomal protein gene that should inhibit this mycotoxin through modification of the toxin's site of action. We have demonstrated that this gene conveys tolerance of the mycotoxin to transgenic tobacco and corn in tissue culture (Harris and Gleddie, U.S. patent #6,060,646; Harris and Gleddie, U.S. CIP filed; Harris and Gleddie, 2001). We have six independent corn lines which are expressing this modified gene constitutively.

During the course of this project, transformation efficiencies improved to the extent that gene transfer in corn is now routine at ECORC at a rate of 100 events/year/technician. ECORC has the only public corn transformation facility in Canada for routine production of transgenics.

In order to direct the expression of genes for resistance, maize gene promoters specific for the tissues susceptible to *Fusarium* attack are needed. We have isolated and characterized a silk-specific promoter (U.S. patent filed). A kernel-specific promoter active during the early part of kernel development, and a fungal-inducible promoter are also being characterized (in progress). We have also initiated a study of the genes turned on in the early infection process in order to learn how the plant responds to *Fusarium* attack. Many

genes belonging to distinct response pathways have been identified and characterized, including novel genes previously uncharacterized. Some of those genes are specifically induced by *Fusarium* in silk, others are induced in both infected silk and ear. These genes will become reference points for future high throughput gene expression studies between susceptible and resistant inbreds.

TABLE OF CONTENTS

FORWARD i

EXECUTIVE SUMMARY ii

INTRODUCTION 1

OBJECTIVES / MILESTONES: 7

MATERIALS AND METHODS 11

RESULTS AND ACCOMPLISHMENTS 25

1) Gene discovery and transformation to improve resistance to *Fusarium* 25

A) Toxin-based Resistance Strategies: Modified *Rpl3* gene 25

B)Toxin-based Resistance Strategies: DON-specific ScFv gene 46

2) Increasing Broad Spectrum Disease Resistance: OXO gene 47

3) Maize Transformation Enhancement 47

4) Enabling Technologies: Promoter Isolation 48

5) Plant/*Fusarium* Interactions 58

REFERENCES 65

REACH AND IMPACT 71

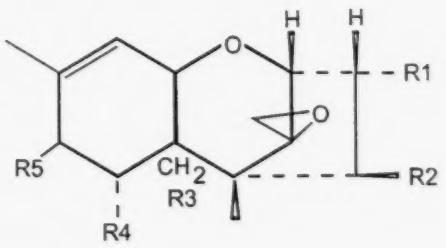
TECHNOLOGY TRANSFER AND COMMUNICATIONS 73

PARTNERSHIPS AND PERSONNEL 77

FINANCIAL REPORT (APRIL 1, 2000 - MARCH 31, 2001) 79

INTRODUCTION

Fusarium graminearum attacks a wide range of plant species including corn (ear and stalk rot), barley, and wheat (head blight/scab). Favorable environmental conditions (conducive temperatures and high humidity) can result in Fusarium epidemics and millions of dollars lost in crop revenues. Disease caused by *Fusarium graminearum* is the single most serious threat to Ontario corn and wheat crops. Mycotoxins produced on the grain are a major concern to livestock producers and the milling industry, and they can have a devastating effect on the value of the crop. The trichothecenes deoxynivalenol (DON) (Figure 1, below) and 15-acetyldeoxynivalenol (15-ADON), as well as zearalenone (Mirocha and Christensen, 1974; Miller et al, 1983), and fusarin C (Farber and Sanders, 1986) are present in maize ears infected with *F. graminearum*. These fungal toxins remain in the contaminated grain after harvest and pose serious health risks to animals and humans who may consume the grain. Canada and the United States have issued guidelines for DON levels in food and feed products. There are several other *Fusarium* species pathogenic on plants, such as *F. sporotrichioides* and *F. culmorum*, which also produce trichothecene mycotoxins.



	R1	R2	R3	R4	R5
Deoxynivalenol	OH	H	OH	OH	=O
Trichodermin	H	Oac	H	H	H

Figure 1. The general structure of the trichothecenes with the specific side chain substitutions present in deoxynivalenol and trichodermin.

Major epidemics occurred in 1982 and 1996 and losses in cash receipts to Ontario corn growers alone are estimated to be over tens of millions of dollars in each of those years. The levels of tolerance in existing corn hybrids were very inadequate in 1996. There are a few natural sources of resistance in germplasm currently being exploited in breeding programs but the mechanism(s) of this resistance is unknown. Mycotoxin-free grain would confer a significant global marketplace advantage to Canadian producers. A major goal of our research program is to produce a more robust and long-lasting resistance by combining genetically enhanced resistance mechanisms with the natural resistance from wild germplasm (tracked with molecular markers) refined by breeders.

One strategy we are developing is based on the accumulated knowledge of the trichothecene toxins' role in the disease process. In addition, isolation and characterization of genes induced early in the infection process was carried out. These genes or their promoters may be modified or enhanced and rapidly incorporated into corn using transformation procedures for fungal tolerance testing. Gene targeting technology most efficient for expression of these resistance genes will also be developed (e.g. corn silk and ear-specific promoters).

Toxin-based Resistance Strategies

The major trichothecene produced by *F. graminearum* is deoxynivalenol (abbreviated as DON, also known as vomitoxin). Trichothecenes are a large family of sesquiterpene epoxides, whose chemical structures vary in the position and number of hydroxylations (see Figure 1 above). Recent field studies with maize and wheat have demonstrated that the presence of trichothecenes enhances the spread of the *Fusarium graminearum* fungal pathogen through the plant tissues and elevates the disease incidence (Desjardins et al. 1996, Harris et al. 1999). These trials were conducted with isogenic strains of *F. graminearum:* either a wild-type pathogenic strain (trichothecene producer, *TRI5**) or a knock-out transgenic strain, in which the first gene in the fungal trichothecene synthetic

pathway was disrupted (trichothecene nonproducer, *TRI5*$^-$). Although the trichothecene-nonproducing strains were able to colonize the wheat heads and corn ears, these infected tissues had reduced disease symptoms and fungal load. Presumably the virulence of *TRI5*$^+$ strains is facilitated through the reduction in plant protein synthesis by trichothecenes produced by the fungi, and the consequential decrease in the plant's production of defence-related proteins. The trichothecene mycotoxins are known to be potent protein synthesis inhibitors in a wide range of eukaryotic organisms including plants and animals and are quite toxic to humans and livestock. As shown in numerous animal and yeast cell culture studies and cell-free systems, trichothecenes bind to a single site on the 60S ribosomal subunit to inhibit protein synthesis in eukaryotes (for review see Feinberg and McLaughlin, 1989).

Thus the effective neutralization of DON or the effect of DON will decrease the virulence or pathogenicity of *Fusarium graminearum*. This could be done in numerous ways, including; by directly decreasing the cells biochemical sensitivity to DON or by introducing a factor that can sequester the DON molecule and inactivate it. Earlier studies in yeast (Grant, 1976; Schultz and Friesen, 1983) demonstrated that a single mutation in the yeast *tcm1* gene could provide tolerance to trichothecenes. The *Tcm1* gene codes for the 60S ribosomal protein L3. This information was used to modify the corresponding rice *Rpl3* gene which was first tested in tobacco and then in corn. For the initial test, we are using a gene promoter which will direct transgene expression throughout the plant at all times. Corn transformed with the rice *Rpl3* gene may possess resistance to *F. graminearum* through a greater tolerance of trichothecenes. A second strategy is designed to sequester the DON molecule. In this case, the gene encoding the active part of a monoclonal antibody, which specifically binds DON, isolated by Ramesh Sinha at ECORC will be used to express an antibody fragment in the plant to neutralize DON.

Oxalate oxidase has been shown to possess general antifungal activity. Sunflower transformed with the oxalate oxidase gene has been reported to exhibit increased

resistance to sclerotinia. Corn has been transformed at ECORC with the oxalate oxidase gene from wheat. Transformed corn plants will be tested for tolerance to *Fusarium*.

The route of *F. graminearum* infection can occur through the silk channels or through wounds to the ear. If promoters could be isolated and developed that could specifically target the expression of defense genes to the susceptible tissues during the window of infection, the energy cost to the plant would be minimized.

The silk is part of the female reproductive organ in corn. It is a long hairy structure attached to the ovary. Pollen grains lodge upon the silk, germinate and the pollen tubes grow down the hair into the silk to reach the ovules (Bonnett, 1948). The corn silk corresponds to the stigma in typical flowers (Heslop-Harrison et al, 1984). The maize silk strands are mainly constituted of one external epidermal layer surrounding many cell layers of parenchyma tissues, and two vascular bundles located within the parenchyma tissue. The vascular bundle areas include pollen transmitting tissues, xylem elements, sieve tube elements and companion cells. The companion cells are morphologically and physiologically associated with the sieve tubes elements (for review: Essau, K., 1965). Companion cells are key players in the phloem loading of assimilates and the synthesis of proteins targeted to enucleate sieve tubes and their functions require transport through plasmodesmata to sieve elements (for review: Sjolund, R.D., 1997).

Fungal spores lodge upon the silk, germinate and the mycelia grow down the strands, either inside or outside, until they reach the ovules where the infection develops further (S. Miller, personal communication). Expression of certain defence genes in silk may increase the resistance of corn to *Fusarium* species. Promoters contain regulatory elements which control gene expression by regulating the transcription of genes. They are typically located in the 5' end of genes, immediately preceding the coding area of the said gene. They frequently contain elements controlling the spatial, temporal and quantitative expression of the genes. To our knowledge, silk-specific promoters are not available to

express transgenes in corn. And it is not known if stigma-specific promoters isolated from dicot species such as *Brassica napus* would be functional in corn. Genes and promoters that have been shown to function primarily in the stigma were identified mainly in a few dicot species. They include proteinase inhibitors from *Nicotiana alata* (Atkinson et al, 1993), chitinases from *Petunia hybrida* (Leung, 1992) and genes involved in the sporophytic self-incompatibility system of *Brassica* (Dzelzkalns et al, 1993; Goring et al, 1993; Nasrallah and Nasrallah, 1993; Robert et al, 1994; Trick and Heizmann, 1992). A gene from soybean, ENOD40(2), has been found to function in root and stigma (Mirabella et al, 1999). Corn silk-specific genes have not been published yet. The only maize silk cDNA libraries from which ESTs have been publically released originated from ECORC.

The plant response to initial attack by *F. graminearum* has not been studied at the molecular level. Most plant/pathogen molecular interactions studied in depth are: 1) in dicots; 2) in systems in which there is a clear gene-for -gene interaction (not broad host pathogens like *F. graminearum*); 3) foliar pathogen (not those infecting reproductive structures). Molecular genetic techniques (such as differential display PCR [Diatchenko et al, 1996]) are available that can be used to identify the specific genes that are turned on by both the plant and the fungi during infection. This will provide us with information as to whether there are genotype specific plant defence responses as well as responses by the fungi to facilitate colonization. Information obtained can be used to develop strategies to engineer resistance. In addition, the plant mRNAs expressed as a result of fungal infection can be used to isolate inducible promoters which respond only to the onset of fungal infection. These promoters will be used to express genes conferring resistance. This gene characterization may also lead to determining the mechanisms of some of the resistance identified by breeders and provide breeders with molecular markers to be able to track this resistance more efficiently through their breeding programs.

The final product in this work is disease-resistant corn for Ontario farmers but the milestones in this project define a variety of deliverables including promoter isolation and information about gene function that have long term value for corn improvement.

OBJECTIVES / MILESTONES

The overall objective for the proposed work is to improve the productivity and quality of Ontario corn varieties through the application of biotechnology; to increase the tolerance to *Fusarium* in corn through gene discovery and transformation.

Gene discovery and transformation for increased resistance to *Fusarium* (ECORC/Agriculture & Agri-Food Canada/Ottawa)

1) Toxin-based Resistance Strategies: Modified *Rpl3* gene and DON-specific ScFv gene (Steve Gleddie, Linda Harris, and John Simmonds)

2) Increasing Broad Spectrum Disease Resistance: OXO gene (John Simmonds)

3) Maize Transformation Enhancement (John Simmonds)

4) Enabling Technologies: Promoter Isolation (Thérèse Ouellet, Jas Singh and John Simmonds)

5) Molecular study of the corn/*Fusarium* interaction (Linda Harris and Thérèse Ouellet)

Milestones for *Fusarium* Biotech Project (ECORC/Ottawa)

1aA	Sept 1998	File an international application under the Patent Cooperation Treaty for the use of the DON-tolerant modified *Rpl3* gene in transformed plants. **Achieved.**
1bB	Apr 1997	Subclone the modified and unmodified plant RPL3 genes into transformation vectors containing gene promoters suitable for insertion into corn. **Achieved.**
1aC	Apr 1999	Test the specificity of 2 different antibody lines raised against the transgenic *Rpl3* protein product. **Achieved.**
1aD	Sept 1999 Apr 2000 Sept 2000	Clone and sequence the genomic copies (~5) of the corn *Rpl3* gene family. **Achieved.** Using gene-specific probes, characterize the expression pattern of the *Rpl3* gene family. Research publication. **Achieved.** (Paper in preparation) Characterize the promoter sequences of the *Rpl3* genes for use in monocot transformation vectors. Research publication. **Achieved.** (Paper in preparation)
1aE		see 3C
1aF	Sept 2000	Express the RPL3 gene on the surface of phage for the purpose of mutagenesis and optimizing binding to DON. **Achieved.**
1bA	Sept 1998 Sept 1999	Sequence the entire heavy chain and light chain variable regions of the anti-DON monoclonal antibody gene. **Achieved.** File a patent on anti-DON monoclonal antibody gene. **Not pursued due to lack of function.**
1bB	Apr 1999	Assemble light and heavy chains into a phage expression vector and test the binding of this to DON. **Achieved.** Research Publication. Construction of plant transformation vectors .**ScFv did not bind DON - project terminated.**
1bC	July 1999	Produce transgenic tobacco expressing the scfv antibody to DON. **ScFv did not bind DON - project terminated.**
1bD		see 3D
2A	Sept 1997	Field trial of T_1 progeny of oxalate oxidase (OXO) transgenics crossed into 12 ECORC inbreds. **Achieved.**

2B	June 1998	Field trial of T_4 progeny of 3 selected inbred lines. **Achieved.**
	Sept 1998	Screen T_4 OXO homozygous lines for *Fusarium* resistance. **Achieved.**
	June 1999	Field trial and *Fusarium* resistance screening of T_7 OXO homozygous elite inbreds. **Achieved.**
3A	Sept 1997	Evaluate tissue culture responses of 12 ECORC inbreds for induction of embryogenic cultures from immature embryos. **Achieved.**
	June 1998	Isolation of TypeII embryogenic culture from CO328X(A188xB73) immature embryos. **Achieved.**
	June 1999	Backcross CO328 into plants regenerated from CO328x(A188xB73) cultures and isolate TypeII cultures from immature embryos. **Achieved.**
	Apr 2001	Continue backcrossing CO328 into plants regenerated from Type II cultures to establish elite germplasm with potential to produce embryogenic cultures. **Achieved.**
3B	June 1999	As in section 2B
3C	Dec 1997	Transform maize A188xB73 cultures with RPL3 genes. **Achieved.**
	June 1998	Demontrate that maize cultures transformed with modified RPL3 are more tolerant to DON. **Achieved.**
	June 1999	Field trial with T_1 RPL3 lines. **Transformation delayed - field trial in 2000.**
	Apr 2001	Field trial and *Fusarium* resistance screening with T_3 RPL3 homozygous lines. **Field trial and *Fusarium* resistance screening with T_1 RPLC4 lines carried out in summer of 2000.** Elite lines with modified RPL3 gene for field evaluation. **T2 RPLC4 lines to be tested in summer 2001.**
3D	Dec 1998	Transform maize A188xB73 cultures with DON antibody gene and establish fertile transgenic plants. **Not pursued - see 1bB**
	Apr 2001	Backcross inbreds into DON transgenics to provide elite lines with DON antibody for field evaluation. **Not pursued - see 1bB**
3	Apr 2001	As 3C
3F	Apr 2001	As 3D
4A	Dec 1997	Construction of a silk-specific cDNA library. **Achieved.**
	Dec 1999	Identification of the kernel and cob tissues initially infected by *Fusarium*. Construction of a kernel and rachis-specific library. **Achieved.**

4B	Dec 1998	Isolate and characterize many silk-specific cDNA clones for their sequence and pattern of expression. **Achieved.**
	Dec 2000	Isolate and characterize kernel and rachis-specific cDNA clones. Select the clones specific to the tissues initially infected by *Fusarium*. **Achieved.**
4C	Apr 1999	Isolate and sequence the genomic copies of selected silk-specific genes. **Achieved.**
4C	Apr 2001	Isolate and sequence the genomic copies of selected kernel and rachis-specific clones. **In progress.**
4D	Apr 2000	Characterize the promoter sequences of selected silk-specific genes by transformation in corn with a marker gene to confirm tissue specificity. **Achieved.**
5A	Sept 1998	Identify, clone and sequence 10 mRNAs induced during the early stages of *Fusarium* infection on corn. **Achieved.**
5B	Sept 1999	Characterize the mRNAs with respect to their genomic origin and gene expression patterns. **Achieved.** Using 5'-RACE technology, isolate and sequence full-length cDNA clones of several of the most promising *Fusarium*-induced mRNAs. Conference presentation. **Achieved.**
5C	Apr 2000	Identify gene inducers and determine the role of several specific *Fusarium*-induced mRNAs in the defense response. **Achieved.** Research publication. **In preparation.**
	Sept 2000	Isolate and sequence the genomic copies of several of the *Fusarium*-induced clones. **Achieved.** Investigate value of filing patents on use of *Fusarium*-induced genes for fungal resistance in plants. **In progress.**
	Sept 2001	Characterize the promoter of some of the *Fusarium*-induced clones. **Achieved - two promoters isolated and characterized.** Investigate value of filing patents on use of *Fusarium*-induced promoters for fungal resistance in plants. **In progress.**
5D	Apr 2001	Transform corn with 1-2 candidate fungal resistance genes.

MATERIALS AND METHODS

1A) Toxin-based resistance strategies: Modified *Rpl3* gene

Site-specific mutagenesis. The wild-type DNA sequence of the *S. cerevisiae Tcm1* gene was obtained from M. Bolotin-Fukuhara of the Yeast Genome Sequencing Project. Upon comparison of the *Tcm1* sequence with the mutant *tcm1* sequence (accession no. J01351), a single basepair change (G to C) was observed (Figure 1). This change converts a tryptophan (*Tcm1*) to a cysteine (*tcm1*) at residue 255 in the proposed yeast RPL3 protein. This is equivalent to residue 258 in the rice RPL3 protein.

The *O. sativa Rpl3* cDNA (originally named T82) was received as a 1368 bp insert in the *Sma*I/*Eco*RI site of pIBI31 (courtesy of A. Kato, Hokkaido University, Japan) (Uchimaya et al, 1992; Nishi et al, 1993). This plasmid was digested with *Xba*I and *Nae*I, yielding a 1722 bp fragment encompassing the *Rpl3* cDNA. This 1722 bp fragment was subcloned into the *Xba*I/*Hpa*I site of the pALTER-Ex1 vector (Promega) and named pALTRPL3. An 18 bp oligomer (5'-GGCTGGATGGCAGGCACC) was used to site-specifically alter a guanine to a cytosine (Promega Altered Sites kit). DNA sequencing confirmed the mutagenesis was successful and the resultant clone was named pALTRPLC4.

Vector Construction. An *Xba*I site (within the vector's multiple cloning site) 5' of the coding region and an *Eco*RI site 8 bp past the rice *Rpl3* TAG stop codon were used to subclone either the unmodified or modified form of the gene into pCAMterX (resultant plasmids named pCARPL3 and pCARPLC4, respectively) (Figure 2). pCAMterX (kindly provided by L. Robert, ECORC) is derived from pBIN19 (Bevan, 1984), with the addition of a 70S (double 35S) cauliflower mosaic virus promoter, multiple cloning site, and nos 3' terminator.

Tobacco Transformation and Selection. pCARPL3 and pCARPLC4 were individually transformed into *Agrobacterium tumefaciens* strain GV3101/pmp90 which was

subsequently used to transform *Nicotiana tabacum* cultivar Delgold and *N. debneyi*. Transformed lines of *N. tabacum* and *N. debneyi* were selected on regeneration medium (Sproule et al, 1991) containing 150 mg/L kanamycin.

Seed harvested from transgenic *N. tabacum* and *N. debneyi* were surface sterilized in 70% Javex solution for 2-3 minutes followed by five rinses in sterile distilled water. They were planted (20 seeds per 60X20 mm petri plate) onto the surface of agar-solidified B_5 medium (Gibco) containing 150 mg/L kanamycin and maintained at 25°C in 16 hr day length of 100 µE/msec. Those seedlings which germinated and remained green following two weeks of selection were transferred to fresh petri plates containing half strength MS medium (Gibco) lacking kanamycin. These plants were maintained inside sterile Magenta containers in a growth room at 25°C in 16 hr day length of 100 µE/msec before transfer to the greenhouse for seed set. Homozygous lines were selected by plating the seed obtained from these plants on B_5 medium containing 150 mg/L kanamycin. Transgenic lines used for subsequent protoplast or cell suspension studies had the presence of the rice *Rpl3* gene confirmed in Southern blot analyses.

RT-PCR Analysis of Expressed Transgenes. Total RNA was isolated using the Trizol reagent (Life Technologies), resuspended at 1 ug/ul, and treated with DNA-Free (Ambion, Inc.) To remove contaminating chromosomal DNA. RT-PCR was carried out using the Superscript One-step RT-PCR for Long Templates System (Life Technologies), as per their suggested protocol, using the Rpl3_3U (5'GTCGCACAGGAAGTTCGA) and Rpl3_1104L (5'AAGCGACCGTGCCCGAAC) primers designed from the rice *Rpl3* cDNA sequence. An aliquot of each RT-PCR reaction was run on a 1% agarose/TBE gel to confirm amplification of the predicted 1.1 kb cDNA sequence. Each RT-PCR reaction was then subjected to three independent restriction digests - *Eco*RV, *Pvu*II, and *Eco*NI - and digests underwent electrophoresis in a 1.2% agarose/TBE gel.

Tobacco Protoplast Isolation. Protoplast isolation from leaf mesophyll cells of *N. tabacum* (*Rpl3* or *Rpl3:c258*) was as described by Sproule et al. (1991). Protoplasts were adjusted to a density of 5×10^4 mL^{-1} with a haemocytometer, in liquid NT medium (Nagata and Takebe, 1971) containing 0.4 M glucose as osmoticum, and either 0, 0.1, 1.0, 5, 10, or 25 ug/ml DON (kindly provided by Dr. Marc Savard, ECORC). A stock solution of deoxynivalenol (DON), produced according to the method of Greenhalgh et al. (1986), was used to adjust the concentration of DON toxin in protoplast cultures. All protoplast cultures were 2 ml of liquid NT medium incubated in sterile 60X15 mm petri plates at 28°C in darkness. After one week in culture, the osmotic concentration of the medium was adjusted by the addition of 0.5 ml NT medium containing 0.3 M glucose, and the protoplast cultures were moved to low light (10 µE msec) at 25°C.

Tobacco Cell Suspension Cultures. Cell suspension cultures from primary transgenic or wild-type tobacco plants were initiated from leaf callus cultures. Two grams of callus was ground in a sterile blender, and the homogenized tissue was used to inoculate 33 ml of liquid MS medium containing 2 mg/L 2,4-D in a sterile 125 ml erlenmeyer flask. Cell suspensions were maintained on an orbital shaker at 150 rpm under a 16 hr day length at 25°C with weekly sub-culture of 5-10 ml of cells into 33 ml of fresh medium.

DON Growth Assays. Growth measurements of cell suspensions of *N. debneyi* (*Rpl3* and *Rpl3:c258*) were taken after the cultures had equilibrated in growth conditions for at least 12 weeks. The measure of weight gain was determined by plating 1 ml of finely filtered cell suspension on sterile Millipore millicell HA filters (0.45um x 30mm diameter) inside sterile 20x60 mm petri dishes containing 2 ml of liquid MS medium with 2 mg/L 2,4-D, supplemented with either 0,10, 25 or 50 ug/ml DON. At 5 day intervals, the fresh weight of each filter unit was determined under aseptic conditions and then the cells were re-cultured on the same medium, with fresh DON added. Cells of both transgenic genotypes were equally capable of growth when transferred to agar-solidified medium supplemented with kanamycin, indicating the stability and presence of the transgenes in these cultures.

RT-PCR of Corn *Rpl3* cDNAs. Total RNA was isolated from maize inbred CO325 silk or seedling tissue using the TRIzol reagent (Gibco/BRL), precipitated and resuspended in water. Total RNA was reverse-transcribed using a 3' Rpl3-specific primer (Rpl3_1104L). RT-PCR was carried out with primers Rpl3_1104L and Rpl3_3U using either regular Taq Polymerase (Perkin Elmer) or the Expand High Fidelity PCR System (Boehringer Mannheim). *Rpl3*-hybridizing PCR products were cloned into the pGEMTeasy vector (Promega).

Genomic Cloning of Corn *Rpl3* Genes. Genomic DNA from inbred CO325 was prepared using a $CsCl_2$ method. The different *Rpl3*-hybridizing *Bgl*II CO325 genomic fragments were size-fractionated on a 0.5% agarose gel, excised, electroeluted, and the presence of the band in the fraction confirmed through Southern analysis. The 5 kb and 6 kb bands were isolated by screening ?ZAP vector (Stratagene) genomic libraries containing the size-fractionated DNA. The 14 kb fraction was cloned into *Bam*HI ?DashII arms (Stratagene) and screened for *Rpl3*-hybridizing plaques. A fourth genomic copy was isolated through genomic PCR, using primers designed from the sequence of one of the four representative *Rpl3* expressed sequences.

Embryogenic Cultures for Particle Bombardment. Immature embryos (1.5-2.0 mm long) of an A188 X B73 derivative were isolated and cultured on medium consisting of N6 salts and vitamins, 2% sucrose, 1mg/L 2,4-D, 25mM proline, 100mg/L vitamin-free casamino acids, 10μM silver nitrate and 0.6% Phytagar (GibcoBRL). Cultures were maintained at 25^0 C in the dark and transferred to fresh medium every 2 weeks.

Particle Bombardment. Particle acceleration was performed with a PDS 1000/He gun (BioRad). Five micrograms of plasmid DNA was precipitated onto 1.6μ gold particles for transient assays and onto 1.0 μ gold particles for stable transformation. A mixture of the following plasmids was used for bombardments: 2.5 μg each of pAct-RPLC4 or pACT-

RPLC3 and pAHC25 (Ubi-GUS-Ubi-BAR). For transformation with gene cassettes 1.25ug of DNA was used.

Embryogenic maize cultures were bombarded 5-7 days after subculturing. Cultures were transferred to medium supplemented with 0.6M mannitol for 2-4 hrs prior to bombardment and were removed to culture medium 24 hrs post-bombardment. Seven days post-bombardment, cultures were transferred to medium containing 2mg/l bialaphos (Meiji Kaisha Ltd, Japan) and then every two weeks until vigorous callus growth was maintained on the herbicide containing medium. For embryo maturation the cultures were transferred to medium containing 0.5mg/L 2,4-D and 10mg/L benzyl adenine and cultured for one week in the dark. They were then transferred to 0.5X salts, hormone-free, sucrose-free medium and cultured at 25^0 C in a 16 h photoperiod ($50\mu Em^{-2}s^{-1}$), from CW fluorescent tubes, for plant development. Rooted plants were established in soil and grown to maturity in greenhouses.

Transgenic Field Trial. Two events (A and D) containing the RPLC4 construct were inoculated for *Fusarium* tolerance testing. Each experiment contained three replicate and each replicate contained 10 plants testing positive (genomic PCR) for the transgene and 10 sib plants testing negative for the transgene. 1 ml of macroconidia inoculum (500,000 spores/ml) was injected into the silk channel (through the husk halfway between the top of the ear and the top of the husk). Kernel inoculations were conducted using an automatic, self-refilling, graduated kernel inoculator. All inoculations were carried out by a single person and all disease ratings were determined blindly by a single individual. The disease rating evaluation is on a scale of 1 to 7 with 7 being the most infected.

1B) <u>Toxin-based resistance strategies: DON-specific ScFv gene</u>

ScFv Cloning. The anti-DON hybridoma cell line was used to isolate polyA RNA which was reverse-transcribed into single stranded DNA. Following second strand DNA

synthesis, we used PCR to amplify the variable domains of the hybridoma light and heavy chain genes. The primers were degenerate murine antibody primers, which have been proven to amplify most light and heavy chain antibodies from hybridomas. The gel-purified light and heavy chain fragments were assembled into a single chain fragment by pull through PCR with a linker fragment between the two chains. The linker fragment codes for a 15 amino acid hinge which allows for the two variable domains to both contact the antigen.

ScFv-DON Binding Assays. The assembled single chain fragment variable (ScFv) gene from the anti-DON monoclonal antibody was then sequenced in a yeast expression vector (Invitrogen). The sequence was of a murine antibody based on database searches, it was in frame, and therefore the vector was transformed into the yeast *Pichea pastoris*. Recombinant ScFv was prepared according to the Invitrogen protocol, and this was evaluated in a DON binding assay. Recombinant ScFv was coated onto ELISA plates using the monoclonal antibody as a positive control protein. Dilute solutions of DON-conjugated to horseradish peroxidase was incubated in each well, and then the residual HRP activity was colorometrically determined. In each assay, the monoclonal antibody was capable of binding to very dilute solutions of DON, whereas the ScFV failed to bind any DON.

3) <u>Maize Transformation Enhancement</u>

Corn Transformation by Biolistics

Embryogenic Cultures. Immature embryos (1.5-2.0 mm long) of an A188 X B73 derivative were isolated and cultured on medium consisting of N6 salts and vitamins, 2% sucrose, 1mg/L 2,4-D, 25mM proline, 100mg/L vitamin-free casamino acids, 10µM silver nitrate and 0.6% Phytagar (GibcoBRL). Cultures were maintained at 25^0 C in the dark and transferred to fresh medium every 2 weeks. New cultures were initiated every 6 months.

Particle Bombardment. Particle acceleration was performed with a PDS 1000/He gun (BioRad). Five micrograms of plasmid DNA was precipitated onto 1.6μ gold particles for transient assays and onto 1.0 μ gold particles for stable transformation. A mixture of the plasmids (2.5 ug each)was used for bombardments. For transformation with gene cassettes 1.25ug of DNA was used.

Embryogenic maize cultures were bombarded 5-7 days after subculturing. Cultures were transferred to medium supplemented with 0.6M mannitol for 2-4 hrs prior to bombardment and were removed to culture medium 24 hrs post-bombardment. Seven days post-bombardment, cultures were transferred to medium containing 2mg/l bialaphos (Meiji Kaisha Ltd, Japan) and then every two weeks until vigorous callus growth was maintained on the herbicide containing medium. For embryo maturation the cultures were transferred to medium containing 0.5mg/L 2,4-D and 10mg/L benzyl adenine and cultured for one week in the dark. They were then transferred to 0.5X salts, hormone-free, sucrose-free medium and cultured at 25^0 C in a 16 h photoperiod ($50\mu Em^{-2}s^{-1}$), from CW fluorescent tubes, for plant development. Rooted plants were established in soil and grown to maturity in greenhouses.

4) Enabling Technologies

Construction of corn silk cDNA library. Corn silk was collected from the field corn plants when the silk emerged from the cob. The silk was frozen immediately in liquid nitrogen and stored in aluminum foil packages at -75° C. Silk RNA as well as RNA from other corn tissues were extracted with Trizol as per instructions by Gibco-BRL Trizol Kit. The cDNA library was constructed in Lambda UNI-ZAP, as per manufacturer's instructions (Stratagene).

Subtractive Supressive hybridization. A subtracted corn silk cDNA probe was isolated by subtracting silk cDNA with seedling cDNA using the PCR-Select cDNA Subtraction Kit (Clontech) and labeling with DIG (Boehringer Mannheim). The subtracted probe contains

an enriched mixture of cDNA's that are expressed in corn silk tissue but not in seedling tissue. Reverse subtracted seedling cDNA probe was isolated by subtracting seedling cDNA with silk cDNA using the same method as above. The reverse-subtracted probe contains a mixture of cDNA's that are expressed in corn seedling tissue but not in silk tissue. Positive clones that hybridize only to the subtracted corn silk probe and NOT to the reverse-subtracted corn seedling probe are isolated. C3 is one of these clones.

Northern blot analyses of C3. Initially, Northern blots were used to confirm whether the C3 clone is indeed silk specific. Corn silk was collected at 0, 2, 4, 6, and 8 days from point of silk emergence (0 day is when corn silk just begins to emerge from the immature cob) and RNA was extracted for hybridization with the C3 probe. 15 ug of total RNA was used per sample. The C3 probe was prepared by excising the C3 clone out of the Lambda UNI-ZAP vector as a pBluescript plasmid containing the C3 cDNA insert. This plasmid was used as the template for a DIG-labeled probe. The resulting C3 DIG-labeled probe was used to hybridize to total RNA (20ug/lane) extracted from corn silk, husk, cob, seedling leaf, seedling root, and Brassica leaf (negative control). A Northern blot containing RNA from a time series of corn silk development was also hybridized with the C3 probe.

Isolation of 5'-regulatory region of C3. The gene regulatory regions of the promoter for the C3 gene was isolated with the method of inverse-PCR. Genomic DNA was purified from corn seedlings and digested with the following enzymes: *Xho*I and *Nde*I (separate digestions). These two enzymes were chosen based on the fact that they cut once within the C3 gene and statistically they are not likely to cut again until 4 Kb upstream and downstream of the gene. The digested genomic DNA fragments were self-circularized under dilute concentrations with T4 DNA ligase and the resulting circularized DNA used as templates in an inverse- PCR reaction. The primer pair c3reverse2 and c3forward2 were designed approximately 150bp from the 5' of the C3 cDNA. The primer pair face away from instead of toward each other like in a typical PCR. As a result, the upstream promoter region was amplified. The *Xho*I digested template yielded an approximately 3 kb band

while the *Nde*I digested template yielded an approximately 2 KB band. Both bands were cut from gel slices and cloned into a TA cloning vector (pGEMT-EASY from Promega.) The resulting clones, pIPCR-XhoII and pIPCR-NdeI were sequenced and the sequences matched each other as well as the orginal C3 cDNA clone. The pIPCR-XhoII clone contained 2Kb of promoter sequence upstream from the C3 gene while the pIPCR-NdeI clone contained 1.2Kb of promoter sequence. Due to an incomplete partial digest, the pIPCR-XhoII clone contained an additional 0.5Kb of terminator sequence downstream of the C3 gene.

Construction of C3-Gus fusion . The complete 2KB promoter region from pIPCR-XhoII was excised and subcloned into a GUS-fusion vector for promoter activity analysis. The construct was named pSilk1 and contains the promoter region from 1 to 1959 (excised with *Ava*II, the closest restriction enzyme site upstream from the ATG start codon of C3 gene). Another construct, pSilk4 was made in which the promoter region was amplified using PCR from pIPCR-XhoII. (Primers ATG and c3up2r). The resulting amplicon contains the promoter region from 208 to 1987 and contains the complete 5'-untranslated region on the cDNA upstream from the ATG start codon of the C3 gene.

Transient Expression of C3-GUS. The above constructs were used for bombardment assays for transient GUS expression. 3mg of 1.6 um gold particles (Bio-Rad) were coated with 5 ug of DNA in the presence of $CaCl_2$ and spermidine and introduced into corn silk tissue (freshly cut, surface sterilized, and maintained on MS agar) by micro-projectile particle bombardment. The bombarded corn silk tissue were assayed for transient GUS expression the next day and GUS enzymatic activity was detected in the form of blue spots upon addition of X-gluc substrate and ferric and ferrous cyanide oxidizers. The pSilk4 construct was found to have much higher GUS expression than the pSilk1 construct. However, the positive control pActin-Gus construct was stronger by a factor of 2 to 3 than the pSilk4 construct (the Actin promoter is a strong constitutive promoter). A promoter-less

GUS construct called pLC.Zprom was used as the negative control and no GUS activity was detected.

Cryosectioning. Silk strands were transferred to the fixing solution (5% formaldehyde, 5% glacial acetic acid, 20% ethanol), followed by a series of transfers to ethanol solutions to dehydrate and preserve the tissues (50% ethanol, 75% ethanol, 100% ethanol) then rehydrate for cryo-sectioning (90% ethanol, 80%, 70%, 60%, 50% ethanol, 25% ethanol), and a final transfer in PBS where sucrose was added to final 30% as a cryoprotectant, let dissolved at 4C for 2 days. Tissue in 30% sucrose were ready for cryo-sectioning. Segments of silk were then frozen in TissueTek O.C.T. compound (Miles Inc., Elkart IN) and 10 µm sections were cut using a Reichert-Jung Cryocut E microtome (Reichert, Vienna). Sections were transferred to glass slides that were pre-treated with Fro-Tissuer Pen (Electron Microscopy Sciences, Fort Washington, PA) and allowed to dry overnight. Sections were counterstained briefly in 0.5% Safranin O (aqueous) for microscopy.

Arabidopsis Transformation. *Arabidopsis thaliana* was transformed using the floral dip method (Clough and Bent, Plant J 16:735-43, 1998 or as described at http://www.cropsci.uiuc.edu/~a-bent/protocol.html)

Construction and screening of maize ear cDNA library. An untreated 8 day post silk emergence *Zea mays* ear (immature kernel and underlying rachis tissue) cDNA library was constructed using the Uni-ZAP XR vector (Stratagene). 14 putative kernel tissue specific clones were isolated after screening approximately 20,000 pfu=s from the above library with kernel-enhanced probes subtracted with seedling cDNA using suppression subtractive PCR (Diatchenko et al, 1996).

Northern analysis of ear-specific clones. Northern blot analysis was performed on total RNA from maize tissue series consisting of leaf, husk, tassel, silk, and ear (10 ug/ lane) at 0 day and 8 days post silk emergence to determine the tissue specificity of the isolated

clones. A complete temporal developmental series was also sampled for maize ear tissue at pre-emergent, 0, 4, 8, 12, 16, 20, and 24 days post silk emergence. It was possible to separate kernels from the underlying rachis at the more mature 12, 16, 20, and 24 day stages; therefore only kernel was used for the RNA extraction instead of the entire ear as was the case for the immature stages before 12 days. Spatial differences in RNA expression were also sampled for 8 day ear from the tip, middle, and bottom regions of the maize ear. Radio-labelled probes were made from the isolated cDNA clone inserts and hybridized to the RNA blots at stringent conditions to confirm the tissue specificity of the putative clones.

5) Plant /*Fusarium* Interactions

Differential Display PCR. The technique of differential display RNA was used to compare subpopulations of transcripts expressed at time zero or 48 hours post-inoculation in maize silk, inoculated with either water or *F. graminearum*. The maize inbreds CO354 (susceptible to gibberella ear rot via *F. graminearum* silk channel inoculation) and CO387 (relatively resistant) were both included in the analysis. Total RNA was isolated from maize silk using the TRIzol reagent (Gibco/BRL), precipitated and resuspended in water.

Differential display (Liang and Pardee, 1992) was performed using the RNAmap kit, version A (Genhunter Corporation), according to manufacturer's instructions. 1ug total RNA was reversed transcribed using anchor primer $T_{12}MC$. 1ul of cDNA was used as template in subsequent PCR reactions using the same anchor primer, $T_{12}MC$, and arbitrary primer #3. PCR buffer and enzyme were supplied by Boehringer Mannheim and products were labeled using ^{33}P-dCTP (1000-3000 Ci/mmol, Amersham). All other components were supplied in the RNAmap kit. The cycling parameters were as follows: 40 cycles of (94^{0}C 30sec, 40^{0}C 2min, 72^{0}C 30sec) followed by a 5 min extension at 72^{0}C. The PCR reactions were run on a 6% denaturing Long Ranger (FMC Bioproducts) sequencing gel

(no stacking effect). The gel was transferred to filter paper, dried, and exposed to KODAK XRP-1 (blue) film for 4 nights at room temperature. PCR reactions containing products that appeared to be differentially expressed were repeated and confirmed. After confirmation, specific bands were excised from the dried gel/filter paper and eluted according to the RNAmap protocol. The excised bands were re-amplified, size-fractionated on agarose gels and purified by electroelution. PCR products were ligated into the pGEM-T easy vector (Promega) overnight at 4°C. Transformations were done using 1ul of each ligation and 60ul of thawed, electrocompetent DH5a cells. Minipreps were done using standard alkaline lysis.

To eliminate false positives, a method similar to that outlined in Callard et al (1994) was used, except digested plasmids transferred to membranes were screened instead of colony dot-blots. A subset of plasmids from each transformation were digested with *Eco*RI, run on 1.2% TBE agarose gels, and bi-directionally transferred to MagnaCharge membrane (Micron Separations Inc.). Each membrane was hybridized with one of two probes: random primed ^{32}P-dCTP-labelled DDRT-PCR reaction at t=0 and at t=48hrs (*F. graminearum*). For these hybridizations, the DDRT-PCR reactions and plasmids all resulted from the same primer combination. Hybridizations were done in 50% formamide/6X SSC at 42°C overnight. Final washes were in 0.1XSSC, 0.1%SDS at 60°C. The membranes were exposed to KODAK XAR film for 3hrs at -70°C.

Southern Analysis. Southern analysis was performed to establish the genomic source (plant or fungal) of the differnetial display clones. Electroeluted *Eco*RI fragments from the differential display clones were random primed using ^{32}P-dCTP (Prime-a-Gene Labelling System; Promega). Southern blots, containing *Eco*RI-digested maize and *F. graminearum* genomic DNA, were pre-hybridized in a solution containing 50% Formamide (deionized), 5x Denhardt's sol'n, 6x SSC, 1% SDS, and 100 µg/ml of sheared herring sperm DNA overnight at 42°C. Hybridizations were done overnight in the same solution with the addition of 5% (w/v) dextran sulphate at 42°C. Final washes were in 0.2XSSC at 55°C or

higher stringency, depending on banding pattern (stringency was increased until single copy hybridization was achieved in most cases). Membranes were exposed to KODAK Biomax film for 1-3 days at -70^{0}C.

Northern Analysis. Denatured total RNA (10µg) was loaded onto 1.3% agarose gels, containing formaldehyde and electrophoresis was performed at 60-80 V for approximately 2 hours depending on gel size. RNA was transferred to MagnaCharge membrane (Micron Separations Inc.) in 10x SSC overnight by capillary action. Probes were labelled via random priming using [a-^{32}P]dCTP (Prime-a-Gene Labelling System; Promega), as per manufacturer's instructions and purified using an Ultrafree-MC microcentrifuge filter unit (Sigma).

Pre-hybridizing and hybridization conditions were identical to those used for Southern blots except that the hybridization solution contained 10% dextran sulfate. Denatured radiolabelled probe was added to the hybridization solution and hybridizations were performed overnight at 42°C. Blots were washed 2x 20 minutes at room temperature in 2x SSC, 0.1% SDS and 2x 20 min at 60°C in 0.1x SSC, 0.1% SDS and exposed overnight at -80°C to BioMaxMS film.

5'-RACE PCR and Sequencing. 5'-RACE PCR (5'-RACE System; Gibco BRL) was preformed according to manufacturer's instruction to acquire a full-length or near-full-length cDNA of several clones. All sequencing was performed by a commercial Licor sequencing service and sequences were compiled and analysed in a Lasergene DNAStar DNA analysis program.

Generacer 5'RACE PCR. 2ug of total silk RNA (CO387 1da p.i.) was used as template and dephosphorylation was done following manufacturers' instructions for the removal of 5' phosphate groups from truncated messages. Precipitated, dephosphorylated RNA was resuspended in 7ul water for the decapping, ligation of RNA oligo, and reverse

transcription that followed the manufacturers protocol. Amplification was carried out using the GeneRacer 5' primer and the gene-specific primer, fi2 L1893 (5' GTC CTC CGT CGC TAG CTT CCA CAT 3').

Touchdown PCR was recommended and used using annealing temperatures of 72°C, 70°C, and 68°C. No PCR product was seen at this point and so a nested PCR was performed as a secondary PCR. Nested PCR used the GeneRacer nested primer and fi2 L1893. PCR products were visible after the second round and cloned into pGEM-T easy (Promega) and sequenced (Thermosequenase deaza-G kit, Amersham Pharmacia Biotech).

Primer Extension. Primer extension was done using the protocol of the Promega Primer Extension system. 10 µg of total RNA (isolated as described previously from CO387 silk tissue, 1 day post-inoculation with *F. graminearum*) was annealed with ^{32}P ?-ATP-labelled primer fi2 L129 (5'-GGT GTC AGC AGA GAG AAC AAT GTA-3') and reverse transcribed with AMV reverse transcriptase.for 30 minutes at 42°C. The T7 sequencing kit (Amersham Pharmacia Biotech) was used to manually sequence 2 µg of fi2 genomic template using the L129 primer. The reactions were run on a 6% LongRanger gel (FMC Bioproducts) for 2 hours (60W), the gel dried and exposed for 1 week at -80°C with Kodak Bio-Max MS film and a Kodak Bio-Max Transcreen-HE intensifying screen.

RESULTS AND ACCOMPLISHMENTS

1) Gene discovery and transformation to improve resistance to *Fusarium*

1A) Toxin-based resistance strategies: Modified *Rpl3* gene

In *Saccharomyces cerevisiae*, tolerance to the trichothecene trichodermin, a termination step inhibitor, was found to be conferred by a single gene, *tcm1* (Schindler et al, 1974; Grant et al, 1976). When the *tcm1* gene was cloned and sequenced, it was shown to code for the ribosomal protein L3 in yeast (Fried and Warner, 1981; Schultz and Friesen, 1983).

Since the trichothecene family of mycotoxins are known eukaryotic protein synthesis inhibitors, we reasoned that if the host target site of action of the trichothecenes could be modified to reduce its susceptibility, the fungus would lose some of its pathogenic advantage and the subsequent spread of the disease, or its severity, would be diminished. In this study we describe the modification of a rice (*Oryza sativa* L.) *Rpl*3 cDNA to resemble the mutation in the yeast *tcm1* gene and the reintroduction of this modified monocot *Rpl3* gene into tobacco which permitted us to measure trichothecene tolerance conferred upon transgenic cells and protoplasts.

The mutation in the yeast gene (*tcm1*) which conferred tolerance to the trichothecene mycotoxin trichodermin was a tryptophan to cysteine substitution at amino acid residue 255 (Figure 2).

Figure 2. cDNA and deduced protein sequence comparison of *S. cerevisiae Tcm1*, *S. cerevisiae tcm1*, and the *O. sativa Rpl3* in the vicinity of the *Tcm1* to *tcm1* mutant change.

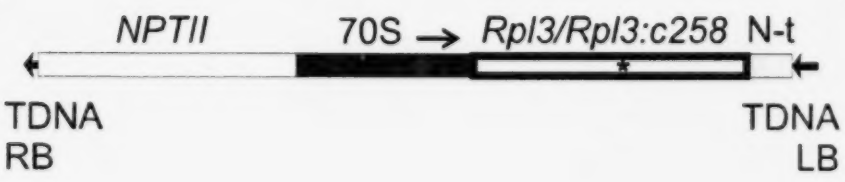
Sc Tcm1, S. cerevisiae (yeast) *Tcm1*; *Sc tcm1, S. cerevisiae tcm1* (accession no. J01351); *Os Rpl3, O. sativa* (rice) *Rpl3*A (accession no. D12630). Nucleotide base and amino acid numbering starts at the initial ATG or methionine. The mutant nucleotide is underlined and in lower case and the relevant amino acids are in bold.

Sc Tcm1	742	AAGGTTGCTTGTATTGGTGCTTGGCATCCAGCCCACGTT												
Sc tcm1	742	AAGGTTGCTTGTATTGGTGCTTG<u>c</u>CATCCAGCCCACGTT												
Os Rpl3	751	AAGGTTGCTTGTATTGGTGCCTGGCATCCAGCCAGGGTG												
Sc Tcm1	248	K	V	A	C	I	G	A	**W**	H	P	A	H	V
Sc tcm1	248	K	V	A	C	I	G	A	**C**	H	P	A	H	V
Os Rpl3	251	K	V	A	C	I	G	A	**W**	H	P	A	R	V

The cDNA sequence of the wild-type rice *Rpl3* gene is highly similar to the yeast *Tcm1* gene and it also encodes a tryptophan at the equivalent amino acid residue 258. This tryptophan residue lies within a region of absolute conservation (amino acids 240-263) not only between the rice and yeast genes, but also with other eukaryotic *Rpl3* genes from mammals (Kuwano and Wool 1992; Simonic et al. 1994) and *Arabidopsis* (Kim et al. 1990).

A Genbank search has failed to uncover any nuclear-encoded eukaryotic *Rpl3* genes from many diverse genera that diverge at all within this region of the protein. The modification of the monocot *Rpl3* gene to produce *Rpl3:c258* was performed and both the wild type rice *Rpl3*, and the variant *Rpl3:c258* were inserted into the binary *Agrobacterium* plant transformation vector derived from pBin19 (Fig. 3).

Figure 3.



In order to determine whether a modified ribosomal protein gene can confer cellular tolerance to trichothecenes, vectors containing either the *Rpl3* or *Rpl3:c258* gene (pCARPL3 and pCARPLC4, respectively) were used to transform tobacco (*Nicotiana tabacum* cv. Delgold) and a wild, diploid species *N. debneyi*. Both genes were transferred into these tobacco species at expected and equal frequencies which suggests that neither rice gene had a negative effect on growth, regeneration, or seed production relative to the other transgene. At least 15 independent transgenic lines of both species were obtained with each vector construct. These plants were self-crossed in the greenhouse, and selfed seed was used to screen for homozygous lines. Southern blots confirmed that all trangenic lines used in these experiments contained single copy insertions of the *Rpl3* or *Rpl3:c258* genes (data not shown). Between 2 and 5 homozygous lines of each species containing each gene were generated and maintained in the growth room or greenhouse.

Expression of rice transgenes in tobacco

The expression of the rice transgenes (*Rpl3* and *Rpl3:258*) was confirmed by RT-PCR analysis on mRNA extracted from leaves of *N. tabacum* and *N. debneyi* plants, using primers designed from the rice sequence. The RT-PCR reactions yielded a strong PCR product of 1.1 kb while a control PCR reaction (no reverse transcriptase) generated a very faint 1.7 kb product from contaminating genomic DNA. The *Arabidopsis Rpl3* gene *ARP2* contains four introns between these two priming sites and would generate a 1.5 kb product. The PCR products were digested with *Eco*RV and *Pvu*II (predicted from tomato *Rpl3* EST sequences to specifically digest within the *Nicotiana Rpl3* genes and not the rice *Rpl3* genes) as well as *Eco*NI (specific for the rice *Rpl3* transgenes). *Eco*RV and *Pvu*II did (and *Eco*NI did not) digest the 1.1 kb RT-PCR product generated from untransformed *Nicotiana* RNA. Because of the specificity of the primers, the rice transgenes were preferentially amplified over the endogenous tobacco *Rpl3* genes. Although this analysis did not measure the level of expression of these transgenes, it is apparent that the transcripts from the rice genes are present in the transgenic leaf RNA. When a Southern blot of the gel

was hybridized with a rice *Rpl3* gene probe, we confirmed that the observed bands originated from the rice *Rpl3* or *Rpl3:258* sequence.

Effect of DON on regeneration from leaf explants

Leaf explants from greenhouse-grown transgenic plants of *N. tabacum* cv. Delgold transformed with either the rice wild-type *Rpl3* gene, or the modified rice gene *Rpl3:c258*, were surface sterilized and cultured on regeneration medium containing either 0, 5 or 10 ug/ml DON. Only leaf explants from plants transformed with the *Rpl3:c258* gene were capable of regeneration and differentiation into shoots with roots, and formed calli on medium containing DON (see Figure 4 below). The presence of 5 ppm DON was not sufficient to cause chlorosis of the leaf explants but was sufficient to inhibit callus formation, shoot primordia development, and plant regeneration on leaf explants of untransformed tobacco or on explants of tobacco transformed with the *Rpl3* gene (not shown).

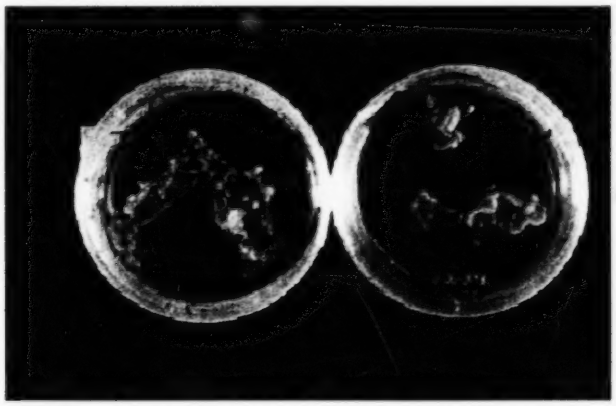


Figure 4. Leaf explants of *N. tabacum* transformed with *Rpl3:c258* (left) or with *Rpl3* (right) following 3 weeks of culture on shoot regeneration medium supplemented with 5 ppm DON. The explants on the left are produing callus, shoot primordia, and appear healthy, while those on the right have not produced any callus, shoots, or shoot primordia.

Plant cell cultures have been used extensively to measure the effect of toxins and inhibitors on growth and development at the tissue, cell, and protoplast levels. The bacterial transgenes *npt II* and *hpt* and the mammalian transgene *dfhr* represent cases of over-expressed genes with the CAMV 35S promoter which conferred upon *N. tabacum* and *N. debneyi* cells and protoplast cultures the ability to grow and differentiate in levels of inhibitors or toxins which were lethal to non-transformed tissues
(Dijak et al. 1991). In the case of the chloroplast protein synthesis inhibitor kanamycin, it is widely used as a selective agent in plant transformation, because the cells and protoplasts of transgenic plants expressing the bacterial *npt* II gene maintain the ability to grow and differentiate in this antibiotic. It has previously been observed that protoplasts cultured in toxic levels of this antibiotic do not die rapidly but rather they fail to regenerate cell walls, fail to divide and form micro-calli (Dijak et al. 1991), which is very similar to the effects of the cytosolic protein synthesis inhibitor DON on tobacco protoplasts reported here. Since dividing protoplasts and cell suspension cultures are metabolically active cells and undergo rapid cell division and expansion they are also highly sensitive to inhibitors of cytosolic and organellar protein synthesis. We have demonstrated in this study that this system is sufficient to measure the effects of the mycotoxin DON on growth and mitotic activity.

Viability of nontransgenic and transgenic protoplasts in the presence of DON

Protoplasts derived from wild-type untransformed plants of both species of tobacco were sensitive to DON at 0.5 to 5 ug/ml in the liquid protoplast culture medium. The effect of DON on these protoplasts was to reduce the ability of protoplasts to reform cell walls, reduce the division frequency (mitotic index of the cells), and reduce the plating efficiency (number of micro colonies formed) of protoplasts relative to those cultured in the absence of DON. The viability of protoplasts of *N. tabacum* was measured microscopically as living vs. dead cells and it was reduced from 70% viable cells at 20 days of culture without DON, to less than 14% viability after 20 days in 5 ug/ml DON. Colonies from protoplasts

containing the rice gene *Rpl3* also did not develop into micro-calli when cultured in liquid medium supplemented with DON, and therefore did not survive transfer to agar-solidified regeneration medium, whereas micro colonies containing *Rpl3:c258* were capable of transfer to solid regeneration medium.

The viability of protoplasts of the genotype *Rpl3:c258* was not significantly affected by culture for 20 days in medium supplemented with 0.1 to 25 ug/ml DON. Whereas the viability of protoplasts containing *Rpl3:c258* in the absence of DON was about 66%, it was reduced to approximately 50% when these protoplasts were cultured in the presence of 25 ug/ml DON. Protoplasts from wild-type tobacco plants cultured in 25 ug/ml DON for three weeks exhibited less than 10% viability while those of transgenic plants with the rice *Rpl3* gene had less than 6% viability. This effect on leaf mesophyll protoplasts was not due to the general effect of each genotype, since in the absence of DON each line had viabilities in protoplast culture medium ranging from 60% to 80% and all experiments were replicated 3 times. The same trend appeared in protoplast cultures from mesophyll cells of wild-type *N. tabacum* or *N. debneyi* which were transformed with either the rice *Rpl3* or *Rpl3:c258* gene. The pronounced differences between genotypes became apparent when protoplasts were cultured in the presence of the mycotoxin DON for extended periods (up to 20 days). When these 20 day old protoplast cultures were plated onto agar-solidified regeneration medium, only those from *Rpl3:c258* plants were capable of consistently forming micro-calli which turned green and continued to differentiate.

Viability and growth rates of cell suspension cultures in the presence of DON

When cell suspension cultures of either *N. tabacum*, *N. debneyi*, or the transgenic lines of these species were established *in vitro*, they were used to measure the growth rates of cells in the presence or absence of the trichothecene DON. Following several months of growth in culture medium lacking DON, the cells were transferred onto liquid medium containing various concentrations of DON. In the presence of DON at either 10, 25 or 50

ug/ml, the growth of wild type cells and trangenic *Rpl3* cells of both species was severely limited. The average weight gain of cells of the three genotypes of *N. debneyi* are shown in Figure 5 after 20 days in culture medium supplemented with various concentrations of DON. The only cell suspension which sustained growth and survived at the highest concentrations of DON (25 and 50 ug/ml) was the cell suspension derived from *Rpl3c:258* plants. In the absence of DON, each of the cell suspensions grew at approximately the same rate over the 20 day interval. The volume of cell suspensions was similarly affected by the presence of DON in the culture medium, and the packed cell volume of wild-type or *Rpl3* cell suspensions in the presence of 10 ug/ml or higher of DON became significantly less than the packed cell volume of *Rpl3c:258* cell suspensions (data not shown).

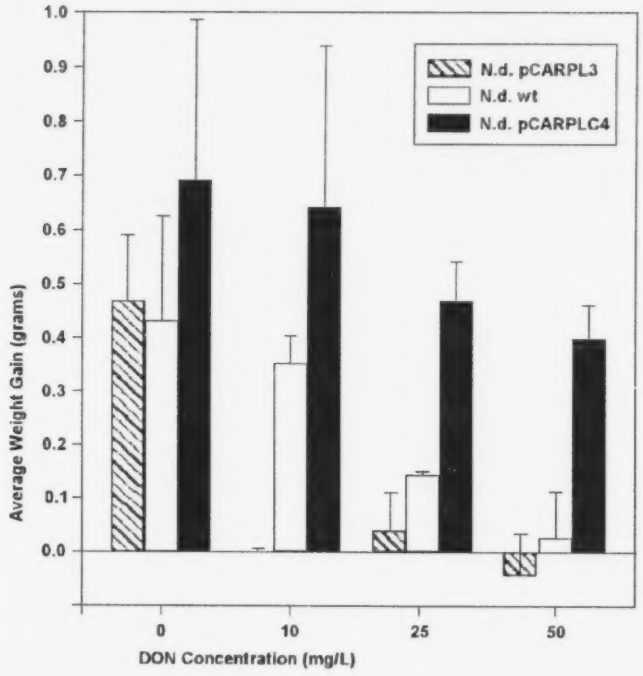


Figure 5. The effect of DON on transgenic *N. debneyi* suspension cultures plated on millicell filters in liquid medium supplemented with various concentrations of DON. The weight gain of cells was measured after 20 days in culture. Bars represent standard errors.

The over expression of the wild-type rice *Rpl3* gene in tobacco did not confer resistance to the mycotoxin DON upon tobacco cells or protoplasts. This suggests that the reason for resistance in the plants which expressed *Rpl3:c258* was the specific substitution of the cysteine for tryptophan in the plant ribosomal protein L3, and not simply an over expression of RPL3. Perhaps this fact may explain the difficulties in finding naturally-occurring germplasm with resistance to the fungal pathogen *F. graminearum*. Since the ribosomal protein L3 is so highly conserved between almost all eukaryotic organisms in the region between amino acids 240-263, this must be an important domain of this ribosomal protein for normal ribosomal function. Presumably most mutations in this region are lethal, and mutations are very rarely if ever found. By modification of the target site of the fungal mycotoxin DON we have shown that transgenic plant cells have elevated tolerance to this toxin.

Cloning of the maize *Rpl3* gene family and expressed cDNA clones

The plant ribosome contains over 70 ribosomal proteins as well as ribosomal RNA sequences. To produce the large amounts of proteins necessary, plant ribosomal protein genes are usually represented by gene families of 2 to 6 copies and most or all of these copies are simultaneously expressed during periods of rapid development and growth. In order to be able to easily distinguish the rice transgene from the endogenous corn *Rpl3* genes, we initiated a study of the corn *Rpl3* gene family. Understanding the regulation of this family may also help in determining the optimal expression of the transgene; one might expect that the right balance of expression would have to be achieved to optimize the proportion of DON-tolerant ribosomes without decreasing yield potential.

A *Bgl*II digest of CO325 genomic DNA yields 5 *Rpl3*-hybridizing bands at 5, 6, 14 (doublet), and >22kb (see Figure 6, below). Three of these copies were cloned by screening size-fractionated genomic bacteriophage libraries. The 5 kb and 14 kb *Bgl*II gene copies have been named *Rpl3A* and *Rpl3B*, respectively. The 6 kb member (named *Rpl3E*) appears

to be a pseudogene as the *Rpl3*-hybridizing sequences are fragmented by DNA insertions. A fourth copy, named *Rpl3C*, was cloned using genomic PCR and PCR primers internal to the coding region. *Rpl3A*, *Rpl3B*, and *Rpl3C* appear intact and functional by sequencing. The fifth genomic copy (*Rpl3D*) is predicted from the sequence of numerous RT-PCR products (see below). *Rpl3A, Rpl3B, and Rpl3C* contain four introns (Table 1). All functional copies and resultant cDNA transcripts can be distinguished by polymorphisms at 24 different nucleotide positions (see Table 1 below).

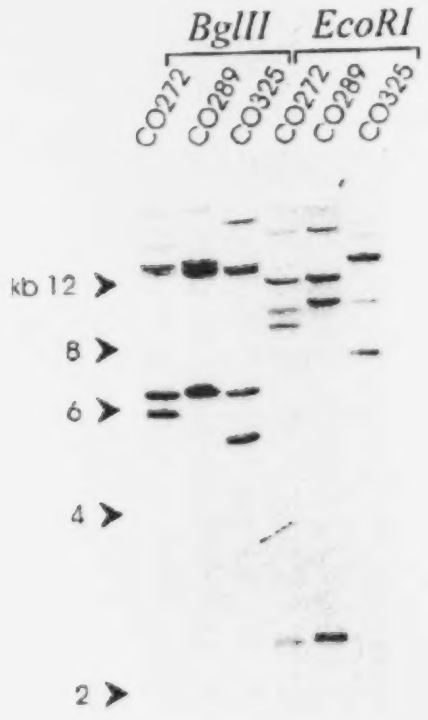


Figure 6. Southern blot of genomic DNA of three different ECORC maize inbreds digested with either *Bgl*II or *Eco*RI and hybridized with the rice *Rpl3* cDNA probe. This Southern illustrates that there are 4-5 gene copies in the *Rpl3* gene family.

Table 1. Polymorphic sites distinguishing four *Rpl3* genomic copies of maize inbred CO325. Numbering is relative to the ATG and relates to nucleotide position in the cDNA (every genomic copy has different intron sizes).

Polymorphism position →	72	1011	1014	2710	3333	5951	5957	6003	6675	6678	6687	7220	7322	7553	7932	8007	8555	8664	9155	9551	9990	10008	10089
Rpl3 maize genomic copy																							
Rpl3A (5kb *Bgl*II gen)	C	T	C	G	C	C	T	T	A	T	T	C	A	G	G	C	T	G	C	C	A	A	T
Rpl3B (14kb *Bgl*II gen)	T	C	T	G	C	T	C	C	G	G	T	G	G	A	C	T	T	G	T	T	C	G	C
Rpl3C (PCR #1)	T	C	T	T	C	T	C	C	G	G	T	G	G	A	C	C	T	A	T	T	C	G	C
Rpl3D (PCR #2)	T	C	T	G	T	T	C	C	G	G	C	G	G	A	C	C	A	G	T	T	C	G	C
		▲		▲	▲													▲					
Intron →		1		2	3													4					
Position →		85		196	501													911					

RT-PCR was carried out on total RNA from maize seedlings (rapidly dividing and developing tissue, relatively high percentage of poly A+ RNA) and from silk (less active tissue, cells elongating, relatively low percentage of poly A+ RNA) using a high fidelity TAQ Polymerase to limit misincorporation of nucleotides. Twenty *Rpl3*-hybridizing products were sequenced from each tissue. These products represented transcripts from all four functional genomic gene copies of *Rpl3*. This survey was not quantitative so we cannot say whether one gene is represented to a greater degree than another, especially as the primers may have covered polymorphic regions, just that all four copies are transcribed in these two tissues.

The corn RPL3 protein is 389 amino acids and shares 94% amino acid sequence identity with the rice RPL3 (see Figure 7 below). In the monocot and dicot plants surveyed, the

region of complete amino acid conservation extends from amino acids 212 to 286, with the exception of position 265 (including *A. thaliana* and oat sequence, not shown). The tryptophan at residue 258 of the rice RPL3 lies within a 20 amino acid region that is completely conserved in plants, fungi, and mammals.

Figure 7. Multiple sequence alignment of several plant RPL3 proteins predicted from cDNA sequences. The wheat, barley, and tomato RPL3 sequences are predicted from a consensus cDNA sequence compiled from at least six EST sequences. The site modified in the protein coded for by rice *Rpl3:258* is indicated by a ▼.

```
Rice      MSHRKFEHPRHGSLGFLPRKRSSRHRGKVKSFPKDDVSKPCHLTSFVGYKAGMTHIVREV   60
Wheat     .....................C........A..R..Q..K....A.L.............
Maize     ..................................R..PK......A.L.............
Barley    .....................C........A..R..Q..K....A.L.............
Tomato    .....................AA.......A.....P....K..A.L.............

Rice      EKPGSKLHKKETCEAVTIIETPPLVIVGLVAYVKTPRGLRSLNSVWAQHLSEEVRRRFYK  120
Wheat     ...................V....I.................T...........D.......
Maize     ..........................................T...................
Barley    ...................V....I.................T...........D.......
Tomato    ...................V....M....V.G.........C..T........DIK.....

Rice      NWCKSKKKAFTKYALKYDSDAGKKEIQMQLEKMKKYASIVRVIAHTQIRKMKGLKQKKAH  180
Wheat     ..........................................TV......................
Maize     ..................EN........L..........VI......................
Barley    ..........................................TV......................
Tomato    ..........L..SK..ET.E...D..A....L....CVI..L.....................

Rice      LMEIQINGGTIADKVDYGYKFFEKEIPVDAVFQKDEMIDIIGVTKGKGYEGVVTRWGVTR  240
Wheat     ......................N.....V.I...................................
Maize     .....V.....................V.....................................
Barley    ......................N.....V.I...................................
Tomato    .....V...S..Q...FA.G....QV.....................................

                                  ▼
Rice      LPRKTHRGLRKVACIGAWHPARVSYTVARAGQNGYHHRTEMNKKVYKIGKSGQESHAACT  300
Wheat     ...................................................V...T.D.S.
Maize     ...................................................A...T.D.S.
Barley    ...................................................V...T.D.S.
Tomato    .........................F.......................L..V.....T.L.

Rice      EFDRTEKDITPMGGFPHYGVVKGDYLMIKGCCVGPKKRVVTLRQSLLKQTSRLALEEIKL  360
Wheat     .......................A.....................................
Maize     ....................I.....................................
Barley    .......................A.....................................
Tomato    ...........I..........E...L.......T.......}

Rice      KFIDTSSKFGHGRFQTTDEKQRFFGKLKA                                  389
Wheat     ..V.....................Y.....
Maize     ............................
Barley    .L....F.....P..D.............
Tomato
```

Modified *Rpl3* gene introduced into maize

Numerous researchers have shown growth inhibition of various monocot tissues by DON. Bruins et al (1993) demonstrated that DON reduces growth of wheat anther-derived callus tissue. DON concentrations of 10 mg/l were sufficient to significantly inhibit growth of mature maize embryos (McLean, 1996). A dose of 100 mg/l DON was lethal to most wheat calli (Menke-Milczarek and Jimny, 1991).

The unmodified *Rpl3* and modified *Rpl3:c258* genes were cloned into a monocot expression vector under the control of the rice actin promoter and intron elements (pCOR13 provided by Dr. R. Wu, U. Cornell, NY) to provide pACTRPL3 and pACTRPLC4, respectively, for constitutive expression in monocots. These constructs were introduced by particle bombardment into cells of embryogenic maize tissue cultures derived from immature F1 embryos of maize A188 X B73. To obtain transgenic lines, each construct was co-bombarded with a selectable herbicide resistant gene-pAHC25 containing the Bar gene (provided by Dr. Peter Quail, UC Berkeley, CA) and phosphinothricin resistant cultures were established. Typically we obtained multiple low copy insertions for both plasmids.

In experiment C41A, the RPLC4 cassette was isolated by restriction digestion with *Kpn*1/*Not*1 for bombardment. This was to reduce vector DNA which can contribute to transgene silencing. Table 2 (below) reports the transformation efficiency. Of 12 plates bombarded with RPLC4+AHC25, 9 produced herbicide resistant calli (75% transformation efficiency). Screening for GUS activity and the presence of RPL4 by PCR demonstrated 84% co-transformation of the transgenes. Similar efficiencies were obtained with RPL3 transformations. For RPLC4, plants were regenerated from each of the 9 responding plates. To date, Southern analyses have identified 8 unique transformations. It is significant that DNA digestion with a non-cutter (*Xho*1) to establish the insertion pattern of the transgenes demonstrated a very simple pattern of insertion for the RPLC4 fragment (Figure 8). Lane A shows 3 inserts of approximately 23, 18 and 9 kb, lane B has a single

4 kb insert, lane E has a single 10 kb insert and lane F has a single 8 kb insert. The expression RPLC4 cassette was 2.9 kb. Plants of each transformation were outcrossed to B73, CO388, CO354 and CO272 . Expression of RPLC4 in the transgenics was confirmed by RT-PCR (Figure 9). Digestion of PCR product with *Eco*N1 releases 300 and 769 fragments from RPLC4 expressing lines. *Pst*1 digestion of PCR product releases 417 and 654 bp fragments of the corn *Rpl3* gene. Six of the 8 RPLC4 transgenic lines expressed RPLC4. T-1 progeny of two high expressing lines (Southern Patterns A and F; Figure 8) outcrossed to B73 and CO388 were advanced to *Fusarium* screening for resistance to silk channel and kernel inoculations in CEF confined field trial 2000. Segregating lines were identified for the presence of RPLC4 by PCR and disease resistance to infection ratings established for transgenic and null lines. All 8 transgenic lines outcrossed to B73 and CO388 have been advanced to the transgenic nursery, segregating transgenic lines will be identified by PCR for continued outcrossing to inbreds. Segregating transgenic lines were identified by PCR and RT-PCR confirmed transgene expression in leaf tissue of the lines used in the *Fusarium* inoculation trials.

Table 2. RPLC4 Transformation (Exp C41A)

Shooting Plate #	# of clones	GUS +	RPLC4 PCR +	% cotrans.	RPLC4 PCR + Reg. Lines	Total # Plants *
3	35	35	26/35	74	18/26	63
4	12	5	5/5	100	5/5	15
7	28	21	18/21	86	6/18	34
8	8	7	7/7	100	3/7	11
9	26	6	4/6	67	2/4	7
10	27	26	23/26	88	21/23	66
11	16	14	8/14	57	5/8	21
12	5	5	4/5	80	4/4	22
9	159	121	97/121	84	64/97	240

Plasmid :
pAct-RPLC4(frag) (1.25 ug)
pAHC25 (5ug)

Date of Bombardment : 12/23/98
Date of first pollination : 7/05399

(*) All lines have given rise to fertile plants, the majority of which have been outcrossed to 4 ECORC inbred lines.

Figure 8. Southern blot of *Xho*1 digested DNA of RPLC4 transformed corn plants

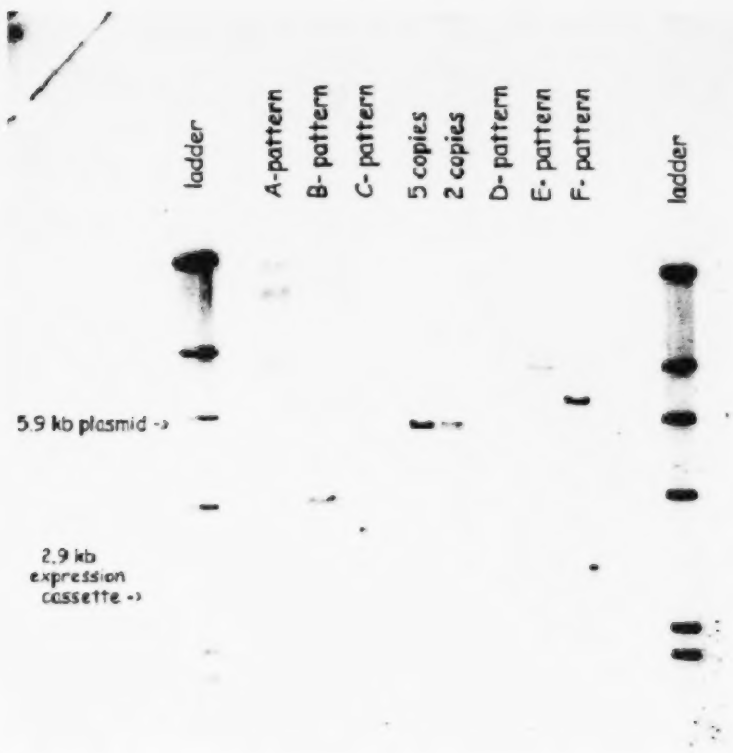
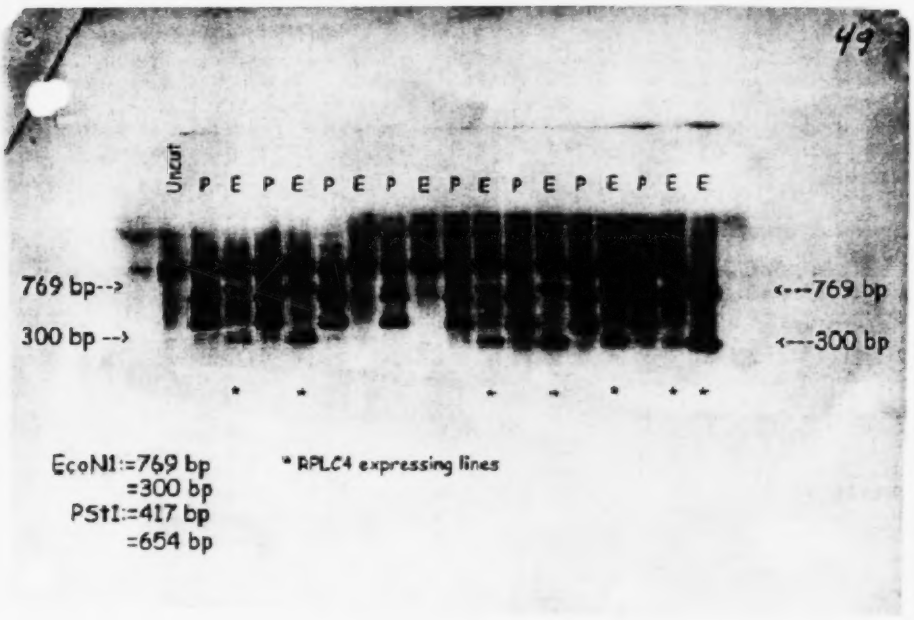


Figure 9. Expression of RPLC4. Southern blot of *Eco*N1 or *Pst*1 digested RT-PCR product from *RPLC4 transgenic corn plants*.



One line of each of RPL3 and RPLC4 was chosen which exhibited a low copy number of the transgene by Southern analysis. Calli from these two lines had undergone identical selection regimes and were of the same age. These two lines were tested for their ability to grow on media containing 0 to 25 mg/l DON. Callus growth on media containing DON showed that the RPLC4 line was substantially more tolerant to DON. RPL3 growth was reduced to 15% of the control by 5 mg/l DON whereas the RPLC4 line was reduced to only 63% of the control value by the same level of DON (see Figure 10 and Table 3 below). To reduce growth of the RPLC4 line to 15% of control values required 50 mg/l DON. This represents a ten-fold increase in tolerance to DON by the RPLC4 callus.

Figure 10. Transgenic maize embryogenic culture (A188xB73) transformed with the wildtype RPL3 (top row) and the RPLC4 (bottom row) genes. DON concentrations are left to right: 0, 5, 10 and 25ppm.

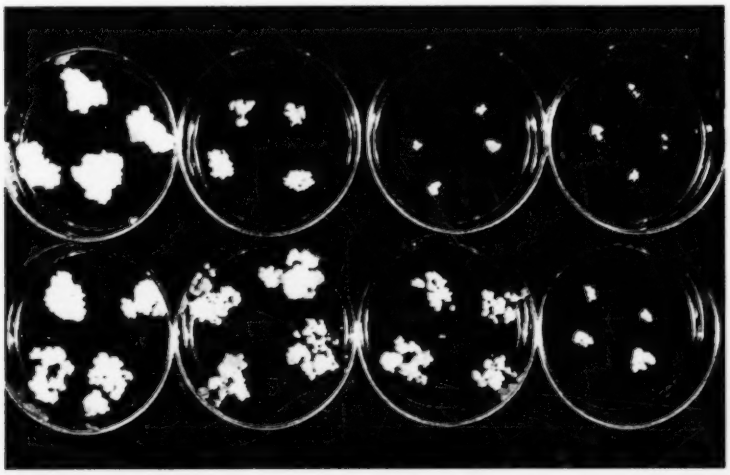


Table 3. Effect of DON on growth of maize embryogenic cultures (A188XB73) transformed with pACTRPL3 and pACTRPLC4
Initial dry wt.: RPL3 = 1.4 mg; RPLC4=1.5 mg. Final dry wt. After 3 weeks culture in the dark at 25°C. 12 explants/treatment.

	DON mg/l				
	0	**5**	**10**	**25**	**50**
pACTRPL3	31.1	4.9	3.3	3.2	2.2
pACTRPLC4	29.8	18.7	13.1	7	4.4

Summer 2000 Field Trial

A transgenic field trial in the summer of 2000 involved the PCR screening and seed amplification of eight events, and silk and kernel *Fusarium* inoculations of two of the RPLC4-expressing events. Table 4 shows the results from the silk channel (500,000 spores/channel) and kernel inoculations of events A and D. Disease rating and DON concentration was determined for each ear. There was no significant difference in either of these two parameters between sib plants with or without the transgene (presence of transgene confirmed by PCR analysis). Transgenic event A exhibited relatively high levels of transgene transcription while event D exhibited moderate levels of transgene transcription. Four of the eight events showed abnormal segregation of the transgene based on PCR screening. In three of them the gene was lost and in one it was lost when used as a pollen donor but retained when used as the female recipient. These results were repeated and confirmed (Table 5) in greenhouse trials during the winter of 2000-01.

Table 4. Transgenic maize field trial results - Summer 2000.

a. Disease Rating (Std error in brackets). Rating scale is from 1 to 7 with 7 being the most infected.

	Silk channel inoculation		Kernel inoculation	
Transgenic line	**PCR+**	**PCR-**	**PCR+**	**PCR-**
895 (A)(XB73)	4.36(0.63)	3.55(0.55)	4.71(0.57)	4.16(0.26)
981(D)(XCO388)	3.42(0.31	3.00(0.23)	3.31(0.3)	3.23(0.37)
985(D)(XB73)	5.45(0.38)	5.19(0.39)	4.28(0.29)	4.00(0.32)

b. DON content analysis in ppm (Std error in brackets)

	Silk channel inoculation		Kernel inoculation	
Transgenic line	**PCR+**	**PCR-**	**PCR+**	**PCR-**
895 (A)(XB73)	554.5(165.2)	399.2(154.5)	376(165.4)	528.1(134.7)
981(D)(XCO388)	117.8(69.9)	24.8(7.0)	46.9(13.7)	43.5(17.9)

Table 5. Segregation of RPLC4 events A, B, E, and H in outcrossed inbred lines

EVENT	T0 RT-PCR	LINE	PCR	n (+/n)
A	+++	T2 AxB73	+	4/7
		T2 B73xA	-	0/28
B	++	T1 BxB73	-	0/13
		T1 B73xB	-	0/25
E	+	T1 ExCO272	-	0/18
I	ND	T1 IxCO272	-	0/20
		T1 CO354xI	-	0/20

Events A, B, E and I were confirmed by Southern blot analysis for T_0 progeny and expression by RT-PCR for A, B and E. The loss of the transgenic event by outcrossing may be due to its insertion at a vital locus affecting embryology so that only segregating null lines survive. In the case of event A, normal segregation was obtained for a maternal transgene but pollen transfer was disrupted.

Events A(maternal), C, D, F and H showed expected 1:1 segregation (by PCR) and were advanced for 2001 field trials. Samples were screened for expression by RT-PCR in the greenhouse in the winter 2000-01 so that only transgene-expressing lines would be included in the field trial. All lines expressed RPLC4 in both leaf and silk tissue (Table 6).

Table 6. Expression of RPLC4 in PCR positive progeny of corn transformed with pAct-Rplc4

EVENT	LINE	RT-PCR LEAF	RT-PCR SILK
A	T2 AxB73	Y	Y
C	T2 CxCO388	Y	Y
D	T2 DxB73	Y	Y
	T2 DxCO388	Y	Y
F	T2 FxB73	Y	Y
	T2 FxCO388	Y	Y
H	T1 HxCO388	Y	Y

The *Fusarium* inoculation block has been planted for the summer of 2001 and includes T1 and T2 generations of segregating populations of plants hemizygous for the transgene. Events D and F in two genetic backgrounds (B73 and CO388) have been established for silk inoculations at three inoculum levels. Events A, C and H will be inoculated with two treatments. The *Fusarium* nursery has been prepared to continue backcrossing the transgenes into inbreds and to maintain seed stocks.

1B) Toxin-based resistance strategies: DON-specific ScFv gene

The PCR generated light and heavy chain fragments from the anti-DON IgG of the hybridoma were cloned and sequenced. These fragments were assembled into a functional ScFv containing a poly (glycine-serine) linker and this sequence is highly homologous to other murine monoclonal antibodies. We assumed that this protein would maintain the same DON binding kinetics as the starting monoclonal IgG. After several ELISA and dot blot assays, we determined that the assembled ScFv did not have any DON binding activity.

In order to account for this discrepancy between the ScFv and the IgG, we decided to obtain the amino terminal sequence of the heavy and light chains of the IgG protein. The chains were separated on SDS polyacrylamide gels and sequenced by Edman degradation. The light chain was N-terminally blocked, and therefore no sequence information was made available. The light chain fragment had created problems for the PCR-based amplification with the primers we used. Several amplification reactions were performed in order to obtain any clonable light chain fragments. The heavy chain however, was easily amplified from the hybridoma, and yielded lots of product. The primary amino acid sequence of the heavy chain IgG protein was determined by sequencing the first 27 amino acids. The sequence of this was compared to the predicted sequence of the PCR product obtained by RT-PCR from the hybridoma mRNA. To our surprise, the sequence of the heavy chain IgG protein was different than the predicted amino acid sequence of the heavy chain variable fragment by three amino acids. We can assume that these differences are PCR artifacts, which may have introduced substitutions at key areas of the ScFv for DON-binding. We will correct these amino acid substitutions by site directed mutagenesis, and re-screen the new ScFv against DON. The possible changes to the heavy chain fragment will be addressed by sequencing cleavage products of the IgG protein to determine what, if any, artifacts of PCR were incorporated into the cloned light chain fragment.

2) Increasing Broad Spectrum Disease Resistance: OXO gene

Eight independent OXO transgenic lines were generated. All T0 lines expressed OXO but in T1, 6 were silenced. The 2 remaining lines were outcrossed to ECORC inbreds. Homozygous and hemizygous T6 lines were established with stable expression on the transgene. Expression in the homozygous line was 3-fold greater than the hemizygous state. In the 1999 field trials, one homozygous line and two hemizygous lines and there corresponding segregating OXO-null lines were field evaluated for resistance to *Fusarium* (silk channel and kernel inoculation), common ear smut and stalk rot. The OXO transgenics did not improve *Fusarium* resistance or stalk rot resistance. A marginal but significant improvement in smut resistance was obtained in the OXO lines.

3) Maize Transformation Enhancement

Critical factors which were significant in improving transformation efficiencies were: maintenance of embryogenic potential of the cultures by attention to visual selection; development of appropriate *in vitro* selection protocols; the employment of OXO as a marker gene to identify transgenic events and development of in-house molecular technologies eg Southerns, PCR, RT-PCR to confirm integration and expression of transgenes. During the course of this project, transformation efficiencies improved to the extent that gene transfer in corn is now routine at a rate of 100 events/year/technician. ECORC has the only public corn transformation facility in Canada for routine production of transgenics.

Data for RPLC4 (Table2) provides an example of the rates of transformation obtained for the RPLC4 gene in this study. Embryogenic cultures were co-bombarded with pRPLC4 or pRPLC3 and pAHC25 (BAR selectable gene). Typically we obtained multiple insertions for both plasmids. In experiment C41A the rplc4 cassette was PCR amplified for bombardment. This was to reduce bacterial DNA which can contribute to transgene

silencing. Of 12 plates bombarded with RPLC4+AHC25, 9 produced herbicide resistant calli. Screening for GUS activity and the presence of RPLC by PCR demonstrated 84% co-transformation of the transgenes. Similar efficiencies were obtained with RPLC3 transformations. For RPLC4, plants were regenerated from each of the 9 responding plates. Southern analyses identified 8 unique transformations. It is significant that DNA digestion with a non-cutter to establish the insertion pattern of the transgenes, demonstrated a very simple pattern of insertion for the RPLC4 fragment (Fig 8).

4) Enabling Technologies: Promoter Isolation

Fusarium graminearum has two modes of entry into the maize ear, either down through the silk channel or through direct wounds to the ear caused by insects or birds. Optimally, one would want to target potential fungal resistance genes to the tissues susceptible to fungal attack. This would minimize the amount of energy required by the plant for this added defense and restrict expression developmentally and spatially. We have isolated both silk-specific and ear-specific clones to permit the isolation of promoters to direct expression primarily in these tissues.

Isolation of a corn silk promoter to target potential fungal resistance genes

A silk cDNA probe subtracted with seedling cDNA and a seedling cDNA probe reverse-subtracted with silk cDNA were used in the differential screening of a complete maize silk cDNA library. 5 out of 23 clones isolated were homologues of a novel gene C3. Analysis of the coding sequence of the gene C3 predicts that the corresponding protein contains a glycine-rich domain. Many proteins with glycine-rich domains have been identified in plants with functions such as RNA-binding proteins (Hirose et al, 1993; Ludevid et al, 1992; Nicolas et al, 1997), cell wall composition/repair proteins (Condit, 1993; Lei et al, 1991; Ryser et al, 1997; Yasuda et al, 1997), proteins present in vascular tissues (Cheng et al, 1996; Parsons and Mattoo, 1994) or yet unidentified, tissue-specific functions (de Oliveira

et al, 1993; Goddemeier et al, 1998; Quigley et al, 1991; Sakuta et al, 1998). Secondary sequence prediction showed the GRP (glycine rich protein) to be a peptide of 187 aa with a N-terminal putative secretory signal peptide followed by a hydrophilic, highly flexible mature protein. The putative peptide has a 40.64% glycine content, followed by tyrosine (8.02%), asparagine (6.95%), serine (5.35%), leucine, proline, and valine (4.81%). Many proteins with glycine-rich domains have been identified in plants with functions such as RNA-binding proteins (Hirose et al, 1993; Ludevid et al, 1992; Nicolas et al, 1997), cell wall composition/repair proteins (Condit, 1993; Lei et al, 1991; Ryser et al, 1997; Yasuda et al, 1997), proteins present in vascular tissues (Cheng et al, 1996; Parsons and Mattoo, 1994) or yet unidentified, tissue-specific functions (de Oliveira et al, 1993; Goddemeier et al, 1998; Quigley et al, 1991; Sakuta et al, 1998).

Northern Blot with C3 gene probe

The gene was confirmed to be silk tissue specific by Northern Blot analysis. The expression level in silk seemed to be developmentally regulated, highest in silk channels at 0 dpe (days post emergence), and decreasing to barely detectable levels at 8 dpe (Figure 11 to right)

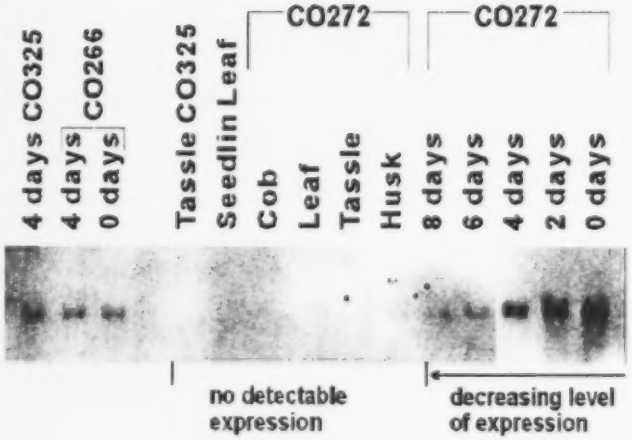


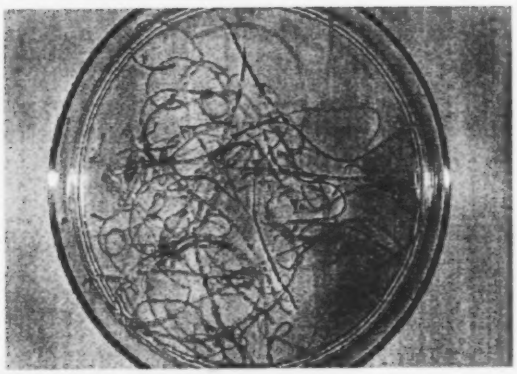
Figure 11. Tissue specificity of C3 expression.

A 2 kb promoter region for the clone was amplified from genomic DNA by Inverse-PCR (Benkel et al, 1996) using gene specific primer pairs designed from the cDNA sequence. The promoter, coding and 3' regions of this gene, C3, showed no significant homology to known coding sequences, except for a region in the first 900 bp of the promoter which includes portions of a Ds transposable element and a cystathione synthase gene. The promoter of the gene C3 has been isolated and transient expression analysis using the promoter fused to the marker gene B-glucuronidase (GUS) for bombardment and stable transformation in corn and *Arabidopsis*. This promoter has the potential to direct preferential expression of transgenes in silk tissues of transformed corn.

Figure 12. Promoter-GUS transcriptional fusion construct: pSilk4-GUS

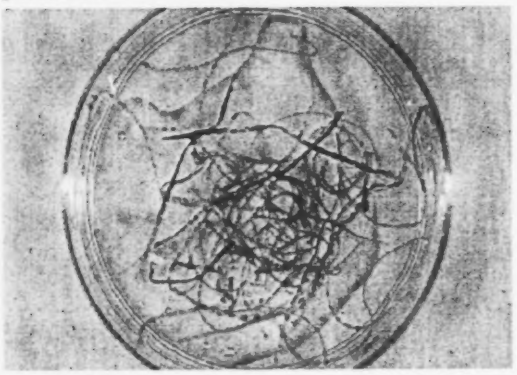
Transient expression of promoter-GUS transcriptional fusion constructs via bombardment of 1-2 dpe silk tissue showed the promoter to be active (Figure 13,14). Stable maize transgenic lines were produced using the same construct. PCR analysis was used to confirm the presence of the transgene. Approximatively 100 plants comprising the transgene were assayed for GUS activity in silk, leaf, husk, tassel and pollen tissues. Corn transgenic lines comprising *pSilk4*-GUS were recovered that exhibited GUS activity in silk strands (Figure 15).

Figure 13



pSilk4-GUS

Figure 14



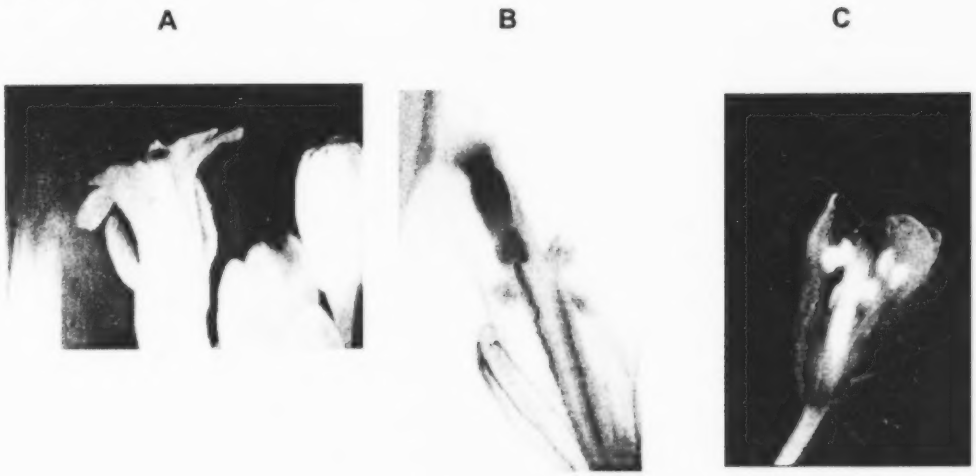
pActin-GUS

Figure 15.

Activity was also detected in pollen grains (not shown). No GUS activity was detected in leaf, cob and tassel tissues. Some of the GUS positive silk strands were processed for cryosection following GUS detection. GUS activity was specifically located in the area of the vascular bundles, including the companion cells (not shown).

Agrobacterium-mediated transformation of *Arabidopsis thaliana* showed the promoter to be active in dicots as well as in monocots. Tissues from *Arabidopsis* plants transformed with either *pSilk4*-Binary-GUS or *pSilk7*-Binary-GUS were assayed for GUS activity in complete seedling, young and mature leaves, flowers and developing fruits. The expression pattern of the GUS reporter gene appeared to be developmentally regulated, with high initial expression in the leaves of young seedlings but eventual confinement to isolated spots along the growing leaf margins and meristem as the plant matures (not shown). The strong activity in the stigmas of the flowers was consistently observed in plants transformed with *pSilk4*-Binary-GUS construct. Stigma specific expression lines were generated (Figure 16a). In addition, some transgenic lines exhibited various level of expression in other floral tissues, including pistil, sepal, petal, anther and pollen (see examples in Figure 16b+c). Preliminary analysis of plants transformed with the *pSilk7*-Binary-GUS construct showed a similar type of expression patterns. This suggested that the region which includes a portion of a Ds element and a truncated cystathione synthase gene is not needed for the C3 promoter activity in plants.

Figure 16.



Silk4promoter- RPLC4 monocot expression vectors were constructed and 14 independent transgenic corn lines were generated. Six were selected and outcrossed to B73. Based on seed set and plant vigour, 4 lines were evaluated for tissue-specific expression of RPLC4 and for inclusion in the 2001 nursery. Segregating T1 pSilk4-RPLC4 lines were identified by PCR and RPLC4 expression evaluated by RT-PCR in four plants from each line. All lines showed expression in leaf tissue. Two also expressed in silk tissue. None were silk specific (Table 7). Strict spatial and temporal regulation by this promoter may be subject to chromosomal location constraints. It should be noted that a similar loss of specificity was observed in the *Arabidopsis* transgenics.

Table 7. Expression of RPLC4 in PCR positive T1 progeny of corn transformed with pSilk4-Rplc4

EVENT	LINE	RT-PCR	
		LEAF	SILK
1600	T1 1600xB73	Y	N
1602	T1 1602x B73	Y	N
1607	T1 1607xB73	Y	Y
1610	T1 1610x B73	Y	Y

Isolation of potential corn ear promoters to target potential fungal resistance genes

Using a kernel-enhanced probe (kernel cDNA subtracted with seedling cDNA), we screened a maize ear cDNA library constructed using RNA from immature kernel and underlying rachis tissue from 8 day post-silk emergence ears. Fourteen putative kernel tissue-specific clones were isolated. Sequence analysis revealed that 6 of the above clones were homologues of germin (oxalate oxidase), for which kernel specificity has already been confirmed in the public database. Of the remaining clones, 2 are highly homologous to 3-deoxy D-arabino- heptulosonate 7-phosphate synthase (DAHP, E.C.4.1.2.15), while one clone each showed homology to S-adenosyl-L-homocysteine hydrolase (SAHH, E.C. 3.3.1.1), UDP-glucose 6-dehydrogenase (E.C. 1.1.1.22), oryzasin (aspartic proteinase), and nucellin-like protein. One putative rachis tissue-specific clone was isolated from the same library using a rachis-enhanced probe (rachis cDNA subtracted with seedling cDNA): caffeoyl-CoA-o-methyltransferase (E.C. 2.1.1.-).

Northern blot analysis was performed on total RNA from maize tissue series consisting of leaf, husk, tassel, silk, and ear (10 ug/ lane) at 0 day and 8 days post silk emergence to determine the tissue specificity of the isolated clones (Figure 17). A complete temporal developmental series was also sampled for maize ear tissue at pre-emergent, 0, 4, 8, 12, 16, 20, and 24 days post silk emergence. It was possible to separate kernels from the underlying rachis at the more mature 12, 16, 20, and 24 day stages; therefore only kernel was used for the RNA extraction instead of the entire ear as was the case for the immature stages before 12 days. Spatial differences in RNA expression were also sampled for 8 day ear from the tip, middle, and bottom regions of the maize ear. Radio-labelled probes were made from the isolated cDNA clone inserts and hybridized to the RNA blots at stringent conditions to confirm the tissue-specificity of the putative clones. Acid phenol was used instead of Trizol for RNA extraction from all the maize tissues due to the high starch content of the more mature kernels. Background levels of hybridization were observed in addition to the true mRNA bands in all tissues. Contaminant genomic DNA could be one

possible reason. Northern blot analysis was repeated for all non-kernel tissues extracted with Trizol, and the background smearing was removed. Efforts are presently underway to re-extract all tissues using the acid phenol method for the sake of consistency, and to remove contaminating genomic DNA with RNase-free DNase.

The nucellin-like proteinase is confirmed to be kernel tissue-specific, with a tightly regulated developmental expression pattern (Figure 17). mRNA transcripts increased from undetectable levels to the strongest detectable level (observed at 4 and 8 days post silk emergence). At 12 days post silk emergence, expression levels decreased to approximately one half of that observed at 8 days, after which the expression level dropped sharply to barely detectable levels at 16, 20, and 24 days post silk emergence. No detectable levels of expression were observed in any other tissue. The expression pattern of the maize nucellin-like proteinase matched that of barley nucellin

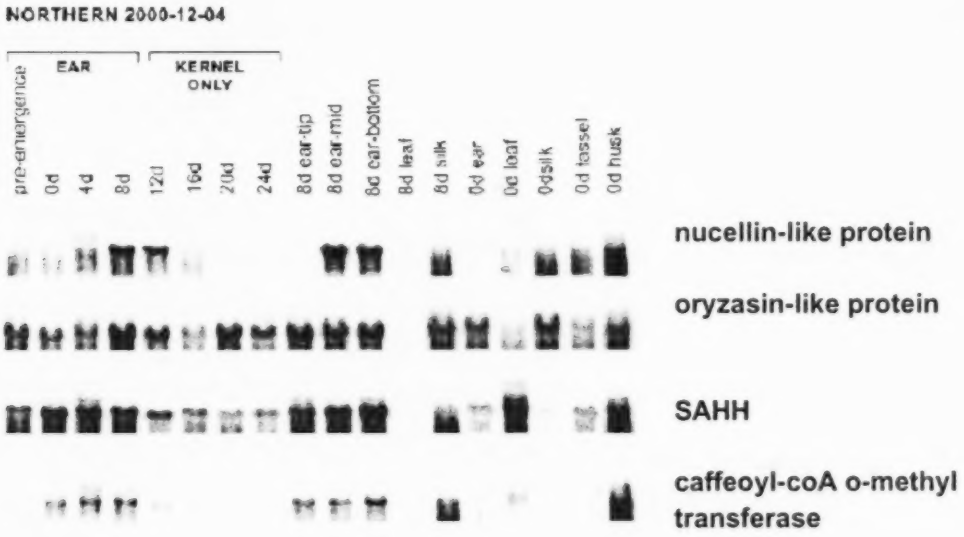


Figure 17. Northern analysis of putative ear-specific clones

(Chen and Foolad, 1997), which was most abundant on ovaries 3-4 days after pollination, but only marginally detectable before pollination or 10 days after pollination. The barley nucellin has been localized to the nucellar cells surrounding the entire embryo sac, and no transcripts were detected in anther, leaf, or root tissue. Based on the high kernel tissue specificity of the nucellin-like proteinase clone, work is currently underway to isolate the 5' gene regulatory region (promoter). The isolated promoter could be useful in driving resistance genes in immature kernel tissue of maize. Based on the spatial and temporal expression pattern observed for barley nucellin, it is likely to be involved in the programmed cell death of the nucellar cells, which are the most common source of apomixis in cereals where one or more cells escape from degeneration and undergo embryogenesis without fertilization (Asker and Jerling, 1992). The isolated promoter could also be useful in silencing expression of endogenous nucellin in maize to induce apomixis in asexual maize seed production.

The oryzasin-like aspartic proteinase expression pattern is not purely kernel-specific (Figure 17). Oryzasin mRNA was present in abundance in kernel RNA from the early (two weeks after flowering) to the middle stages (4 weeks after flowering) of seed ripening and is speculated to function in the processing of seed storage proteins (Asakura et al, 1995). Oryzasin mRNA was strongly expressed in rice leaves at 5 days after fertilization, but was reduced to undetectable levels at 7 days. This closely matches the expression level we observed in maize (weak expression in leaves at 0 days post silk emergence, and undetectable at 8 days.) Promoter isolation for the maize oryzasin-like proteinase clone has not been initiated. While oryzasin has a wider temporal expression pattern in ripening seed, the tissue specificity of expression is not tightly controlled.

SAHH expression is not kernel tissue specific. Although weak in leaf tissue, detectable levels of expression were observed in all tissues tested.

The caffeoyl-CoA-o-methyltransferase clone was not rachis-tissue specific. RNA transcripts were detected in 0, 4, and 8 day ear (kernel + rachis) as well as 12, 16, 20, and 24 day kernels. The role of caffeoyl-CoA-o-methyltransferase in lignin synthesis does not suggest a spatial expression pattern confined to only the kernel or rachis tissues.

The predicted functions of DAHP and UDP-glucose 6-dehydrogenase did not suggest a kernel specific localization, and the above two clones were not subjected to Northern blot analysis.

5) Plant/*Fusarium* Interactions

The molecular interactions between maize and *F. graminearum* were have been studied during early infection of the silk channel in a susceptible (CO354) and a resistant (CO387) inbred. Briefly, *F. graminearum* was inoculated into the silk channel of the maize ears 4 to 6 days after silk emergence. Mock inoculations (with sterile fungal media) were also performed in parallel. Differential Display RT-PCR was used to identify genes, from maize and *F. graminearum*, that are elicited in the early stages of infection of maize silk by the fungus. DNA fragments showing differential expression during early infection were cloned and characterized using sequencing, as well as Northern and Southern analyses.

Sixteen of the cloned fragments showed a differential pattern of expression between time 0 and 48 h after fungal inoculation, as well as between fungal and mock-inoculated samples, when using the differential display procedure. Seven of the 14 maize clones (two of the sixteen clones originated from the fungus) have been confirmed to be induced or up-regulated early in infection by Northern blot analysis (Table 8). Focusing on a few genes, we have used 5' RACE PCR to obtain near full-length cDNA clones and conducted Northern analyses to further characterize their gene expression (Tables 5 and 6, Figures 18 to 20). The expression analyses have included time course studies, tissue specificity,

and testing for induction by other fungal pathogens (e.g.*Fusarium verticillioides*, causal agent of Fusarium ear rot and fumonisin-producer) and inducing agents (e.g. salicylic acid).

The gene fiB is induced in silk by *Fusarium graminearum* about 24 to 36 hrs after the inoculation, and remains active over many days (Figure 19). The expression pattern after treatment with salicilic acid (SA), an inducer of many defense genes, suggests that fiB is activated by, or is part of a pathway activated through salicilic acid . The function of fiB, or of any homologue in other species, has not been identified yet.

fiD is the maize homologue of GSPE, a protein with unknown function which is very well conserved throughout evolution, from bacteria to plants, and has been shown to be essential in *E. coli.* This gene is induced as early as 6 hrs after inoculation by *F. graminearum* in silk and ear, and will remain active over many days (Figure 18). It is also induced by *F. verticillioides.* Salicilic acid treatment produced only a transient activation at about 6 hrs, suggesting that it can be involved in the initial induction of fiD, however it is cannot maintain by itself the activity of the gene.

The gene fi8 has a unique pattern of expression in maize silk (Figure 20). It can be transiently induced within the first 6 hours of treatment by *F. graminearum* inoculation as well as by simple wounding (mock inoculation with media or water), suggesting that this initial induction is a wounding response. Then there is an additional induction only in presence of *Fusarium* over the following 1 to 2 days. This *Fusarium*-specific induction shows oscillation which may be associated with light periods. Similar expression trends were observed with samples from three growing seasons. Salicylic acid does not seems to play a role in fi8 expression as the induction observed with SA is similar to the one observed with mock treatment. The function of fi8 is not known, however its protein has some homology with a potential circadian clock protein from *Arabidopsis.*

Table 8. Summary of Differential Display Clones

Clone	Size (bp)	Southern Analysis (copy number)	Blast Identity	5' RACE	Northern Analysis (total RNA)		
					48h/H_20	0h/*Fus*	48h/*Fus*
pfi1	354	*Z. mays* (3)	no		-	-	+
pfi2	560	*Z. mays* (1)	yes	✔(2.3 kb)	-	-	++
pfi3	451	*F. graminearum* (1)	no		-	-	-
pfi4	380	*Z. mays* (1-4)	cytochrome P450 homology		-	-	-
pfi5	226	*Z. mays* (2-5)	no		-	-	-
pfi6	381	*Z. mays* (1)	no		-	-	+
pfi7	354	*Z. mays* (1-3)	receptor-like protein kinase	✔(0.8kb)	+	+	++
pfi8	298	*Z. mays* (1)	maize EST match	✔(1.1kb)	+	-	++
pfi9	332	*Z. mays* (1)	sorghum EST		+	-	++
pfi10	263	*Z. mays* (1)	no		++	+	++
pfi11	350	*Z. mays* (1)	yes		-	-	-
pfiB	497	*Z. mays* (1)	maize EST match	✔(0.75kb)	-	-	++
pfiD	552	*Z. mays* (1)	GSPE protein	✔(2.4kb)	+	+	++
pfiE	439	*Z. mays* (2)	no		-	-	-
pfiF	434	*F. graminearum* (1)	mitochondrial ATPase subunit		-$	-	-
pfiI	265	*Z. mays* (1)	no		-	-	-

-: no detectable hybridization; +: weak hybridization; ++: strong hybridization.
$ Northern analysis: detected in *F. graminearum* mycelial total RNA

Table 9. Summary of Detailed Notrhtern Analysis for Some Maize Clones induced by *F. graminearum*

Tissue	Silk					Eartip			Leaf	Tassel
Treatment ➡	FG	FV	SA	Mock	None	FG	Mock	None	None	None
Clone										
pfi2	++++	++++	+/-	-	-	+++	-	-	-	++
pfi8	++++	+++	+	++	+	-	-	-	-	-
pfiB	+++	++	++	+	+	+	+	+	+	+
pfiD	++++	+++	++	+	-	++++	+	+	+++	+++

All plants were inoculated/treated through the silk channel and time course experiments were performed for each treatment. Maximum signal intensity reached during the treatment is indicated. *F. verticillioides* (FV) is a fumonisin-producing fungus also causing Fusarium head blight in maize. FG, *F. graminearum*; SA, salicylic acid; -, no detectable hybridization; + to ++++, weak to very strong hybridization.

Figure 18. Expression of fiD clone in silk (A, C) and ear tip (B) after silk channel inoculation with *F. graminearum* (FG), *F. verticillioides* (FV), 1 mM salicilic acid (SA) or sterile media (mock). Expression in non-treated tissues is also shown (C). For non-treated samples, days are from silk emergence.

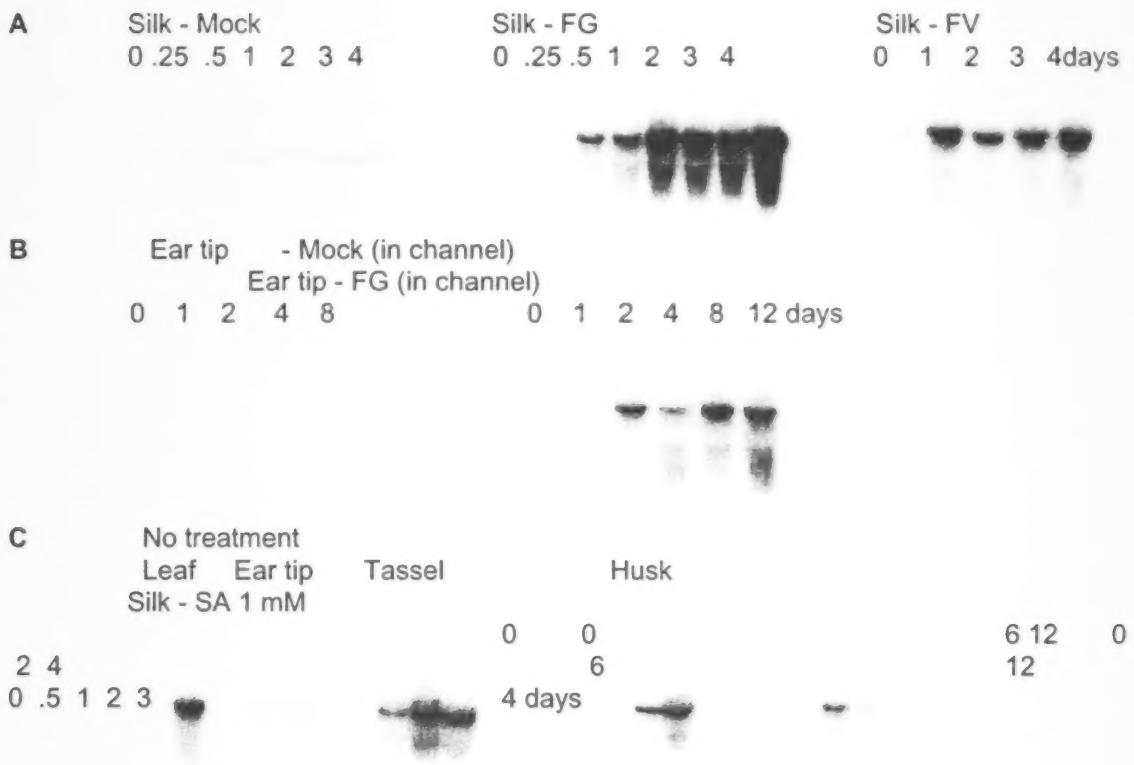
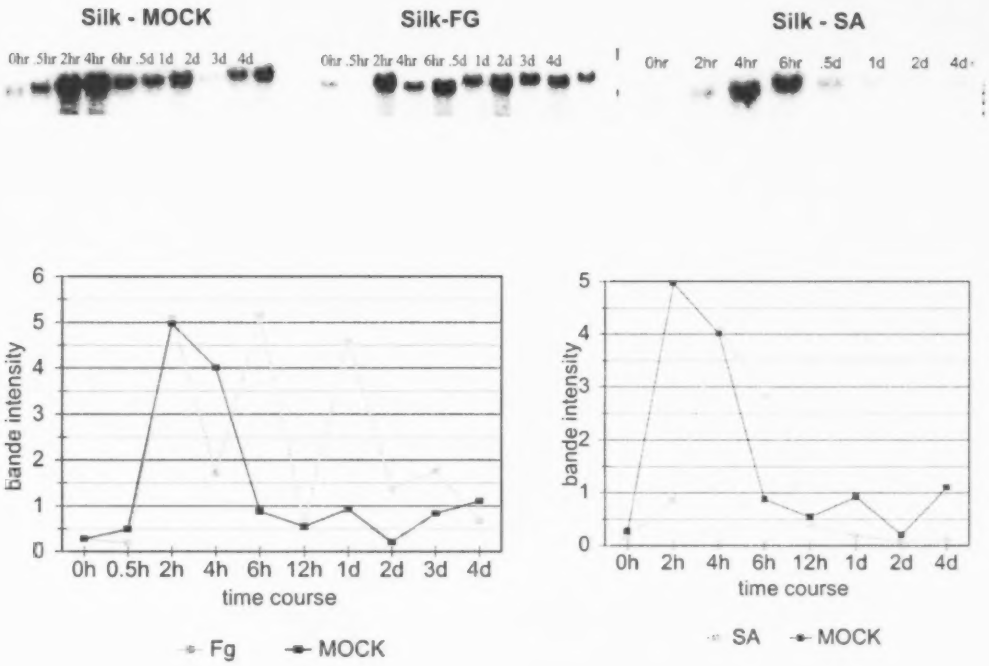


Figure 19. Time course of expression of pfiB clone in the CO 354 inbred silk channel-inoculated with sterile fungal media (mock), *Fusarium graminearum* (Fg), or with salicylic acid (SA). All plants were inoculated into the silk channel and time courses were performed for each inoculum.

Figure 20. Time course of expression of pfi8 clone in the CO 387 inbred inoculated with sterile fungal media (Mock), *Fusarium graminearum* (FG), or with salicylic acid (SA). The graphs show the expression profile of the pfi8 clone after normalisation using the actin gene as a probe in order to compensate for the variations in amount of RNA between sample.


Silk - MOCK
0hr .5hr 2hr 4hr 6hr .5d 1d 2d 3d 4d
Silk-FG
0hr .5hr 2hr 4hr 6hr .5d 1d 2d 3d 4d
Silk - SA
0hr 2hr 4hr 6hr .5d 1d 2d 4d
bande intensity
0 1 2 3 4 5 6
0h 0.5h 2h 4h 6h 12h 1d 2d 3d 4d
time course
Fg
MOCK
bande intensity
0 1 2 3 4 5
0h 2h 4h 6h 12h 1d 2d 4d
time course
SA
MOCK

REFERENCES

Asakura T et al, 1995. Eur. J. Biochem. 232:77-83.

Asker SE, Jerling L. 1992. Apomixis in Plants. CRC Press, Boca Raton, FL.

Atkinson A.H., Heath, R.L., Simpson, R.J., Clarke, A.E., Anderson, M.A. 1993. Plant Cell 5: 203-213.

Bevan, M. 1984. Agrobacterium vectors for plant transformation. Nucl. Acids Res. 12:8711-8721.

Bonnett, O.T. 1948. Ear and tassel development in maize. Annals of the Missouri Botanical Garden 35, 269-287.

Bruins et al. 1993. Plant Sci. 94:195-206.

Callard, D., B. Lescure, and L. Mazzolini. 1994. A method for the elimination of false positives generated by the mRNA differential display technique. Biotechniques 16:1096-1103.

Carrasco, L., M. Barbacid, D. Vazquez. 1973. The trichodermin group of antibiotics, inhibitors of peptide bond formation by eukaryotic ribosomes. Biochim. Biophys. Acta 312:368-376.

Chen and Dellaporta. 1994. The Maize Handbook, pp526-527.

Chen, F., Foolad, M.R. 1997. Plant Mol. Biol. 35:821-831.

Cheng, S.H., Keller, B., Condit, C.M. 1996. Common occurrence of homologues of petunia glycine-rich protein-1 among plants. Plant Mol. Biol. 31, 163-168.

Condit, C.M. 1993. Developmental expression and localization of petunia glycine-rich protein 1. Plant Cell 5, 277-288.

De Oliveira, D.E., Franco, L.O., Simoens, C., Seurinck, J., Coppoeters, J., Botterman, J., Van Montagu, M. 1993. Inflorescence-specific genes from *Arabidopsis thaliana* encoding glycine-rich proteins. Plant J. 3, 495-507.

Desjardins A.E., T.M. Hohn. 1997. Mycotoxins in plant pathogenesis. Mol. Plant-Microbe Interact. 10:147-152.

Desjardins, A.E., R.H.Proctor, G. Bai, S.P. McCormick, G. Shaner, G. Buechley, T.M. Hohn. 1996. Reduced virulence of trichothecene-nonproducing mutants of *Gibberella zeae* in wheat field tests. Mol. Plant-Microbe Interact. 9:775-781.

Diatchenko, L. *et al, PNAS*, 93:6025-6030, 1996

Dijak, M., A. Sproule, W. Keller, R. Pandeya, S. Gleddie. 1991. Transformation of *Nicotiana tabacum*, *N. debneyi*, and *N. rustica*: Inheritance and protoplast expression of antibiotic resistance. Plant Cell Tissue Organ Cult. 25:189-197.

Dzelzalns, V.A., Thorsness, M.K., Dwyer, K.G., Baxter, J.S., Balent, M.A., Nasrallah, M.E., Nasrallah, J.B. 1993. Distinct cis-acting elements direct pistil-specific and pollen-specific activity of the *Brassica* S locus glycoprotein gene promoter. Plant Cell 5, 855-863.

Essau, K. 1965. Plant anatomy. John Wiley & Sons, ed. New York.

Farber, J.M., G.W. Sanders. 1986. Production of fusarin C by *Fusarium spp.* J. Agric. Food Chem. 34:963-966.

Feinberg, B., C.S. McLaughlin. 1989. Biochemical mechanism of action of trichothecene mycotoxins. In: Trichothecene mycotoxicosis: Pathophysiologic effects. Edited by Beasley, V.R.: CRC Press, Inc, Boca Raton, Florida, p.27-35.

Fried, H.M., J.R. Warner. 1981. Cloning of yeast gene for trichodermin resistance and ribosomal protein L3. Proc. Natl. Acad. Sci. USA 78:238-242.

Goddemeier, M.L., Wulff, D., Feix, G. 1998. Root-specific expression of a *Zea mays* gene encoding a novel glycine-rich protein, zmGRP3. Plant Mol. Biol. 36, 799-802.

Goring, D.R., Glavin, T.L., Schafer, U., Rothstein, S.J. 1993. Plant Cell 5, 531-539.

Grant, P.G., D. Schindler and J.E. Davies. 1976. Mapping of trichodermin resistance in *Saccharomyces cerevisiae*: A genetic locus for a component of the 60S ribosomal subunit. Genetics 83:667-673.

Greenhalgh, R., D. Levandier, W. Adams, J.D. Miller, B.A. Blackwell, A.J. McAlees, A. Taylor. 1986. Production and characterization of deoxynivalenol and other secondary

metabolites of *Fusarium culmorum* (CMI 14764, HLX 1503). J. Agric. Food Chem. 34:98-102.

Harris, L.J., A.E. Desjardins, R.D. Plattner, P. Nicholson, G. Butler, J.C. Young, G. Weston, R.H. Proctor, T.M. Hohn. 1999. Possible role of trichothecene mycotoxins in virulence of *Fusarium graminearum* on maize. Plant Dis. 83:954-960.

Heslop-Harrison, Y., Reger, B.J., Heslop-Harrison, J. 1984. The pollen-stigma interaction in the grasses. Tissue organisation and cytochemistry of the stigma ("silk") of *Zea mays* L. Acta Bot. Neerl. 33, 81-99.

Hirose, T., Sugita, M., Sugiura, M. 1993. CDNA structure, expression and nucleic acid-binding properties of three RNA-binding proteins in tobacco: occurrence of tissue-specific alternative splicing. Nucleic Acids Res. 21, 3981-3987.

Hood, E.E., G.L. Helmer, R.T. Fraley, and M.D. Chilton.1986. The hypervirulence of *Agrobcterium tumefaciens* A281 is encoded in a region of pTiBo542 outside of T-DNA. J. Bacteriol. 168:1291-1301.

Jefferson, R.A. 1987. Assaying chimeric genes in plants: the GUS gene fusion system. Plant Mol Biol Reo 5: 387-405.

Kim, Y., H. Zhang, R.L. Scholl. 1990. Two evolutionary divergent genes encode a cytoplasmic ribosomal protein of *Arabidopsis thaliana*. Gene 93:177-182.

Kuwano, Y., I.G. Wool.1992. Primary structure of rat ribosomal protein L3. Biochem. Biophys. Res. Commun. 187:58-64.

Lazo, G.R., P.A. Stein and R.A. Ludwig.1991. A DNA transformation-competent Arabidopsis genomic liberary in Agrobacterium. Bio/Technology 9, 963-967.

Lei, M., Wu, R. 1991. A novel glycine-rich cell wall protein gene in rice. Plant Mol Biol. 16, 17-198.

Leung, D.W.M. 1992. Phytochemistry 313, 1899-1900.

Liang, P. and A.B. Pardee. 1992. Differential display of eukaryotic messenger RNA by means of the polymerase chain reaction. Science 257:967-971.

Ludevid, M.D., Freire, M.A., Gomez, J., Burd, C.G., Albericio, F., Giralt, E., Dreyfuss, G., Pages, M. 1992. RNA binding characteristics of a 16 kDa glycine-rich protein from maize. Plant J. 2, 999-1003.

McLean 1996. Mycopathologia 132:173-183.

Menke-Milczarek and Jimny 1991. Mycotox. Res. 7:146-149

Miller, J.D., J.C. Young, H.L. Trenholm. 1983. *Fusarium* toxins in field corn. I. Time course of fungal growth and production of deoxynivalenol and other mycotoxins. Can. J. Bot. 61:3080-3087.

Mirabella, R., Martirani, L., Lambert, A., Iaccarino, M., Chiurazzi, M. The soybean ENOD40(2) promoter is active in *Arabidopsis thaliana* and is temporally and spatially regulated. Plant Mol Biol. 39, 177-181.

Mirocha, C.J., C.M. Christensen. 1974. Fungus metabolites toxic to animals. Ann. Rev. Phytopathol. 12:303-330.

Murashige, T., F. Skoog. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant. 15:473-497.

Nagata, T., I. Takebe. 1971. Plating of isolated tobacco mesophyll protoplasts on agar medium. Planta 99:12-20.

Nasrallah, J.B., Nasrallah, M.E. 1993. Plant cell 5, 1325-1335.

Nicolas, C., Rodriguez, D., Poulsen, F., Eriksen, E.N., Nicolas, G. 1997. The expression of an absicisic acid-responsive glycine-rich protein coincides with the level of seed dormancy in *Fagus sylvatica*. Plant Cell Physiol. 38, 1303-1310.

Nishi, R., S. Kidou, H. Uchimiya, A. Kato. 1993. The primary structure of two proteins from the large ribosomal subunit of rice. Biochim. Biophys. Acta 1216:110-112.

Parsons, B.L., Mattoo, A.K. 1994. A wound-repressible glycine-rich protein transcript is enriched in vascular bundles of tomato fruit and stem. Plant Cell Physiol. 35, 27-35.

Quigley, F., Villiot, M.L., Mache, R. 1991. Nucleotide sequence and expression of a novel glycine-rich protein gene from *Arabidopsis thaliana*. Plant Mol. Biol. 17, 949-952.

Rotino, G.L., S. Gleddie. 1990. Transformation of eggplant (*Solanum melongena* L.) using a binary *Agrobacterium tumefaciens* vector. Plant Cell Rep. 9:26-29.

Ryser, U., Schorderet, M., Zhao, G.F., Studer, D., Ruel, K., Hauf, G., Keller, B. Structural cell-wall proteins in protozylem development: evidence for a repair process mediated by a glycine-rich protein. Plant J. 12, 97-111.

Satuka, C., Oda, A., Yamakawa, S., Satoh, S. 1998. Root-specific expression of genes for novel glycine-rich proteins cloned by use of an antiserum against xylem sap proteins of cucumber. Plant Cell Physiol. 39, 1330-1336.

Schindler, D., P.G. Grant, J. Davies. 1974. Trichodermin resistance - mutation affecting eukaryotic ribosomes. Nature 248:535-536.

Schultz, L.D., J.D. Friesen. 1983. Nucleotide sequence of the *tcm1* gene (ribosomal protein L3) of *Saccharomyces cerevisiae*. J. Bacteriol. 155:8-14.

Simonic, T., G. Simona, F. Giussani, M. Malcovati, S. Ronchi, M.L. Tenchini. 1994. cDNA sequence for bovine ribosomal protein L3 carrying a bipartite nuclear targeting motif, identified alos in many other ribosomal proteins. Biochem. Biophys. Acta 1219:706-710.

Sjolund, R.D., 1997, The phloem sieve element: a river runs through it. Plant Cell 9, pp1137-1146

Sproule, A., P. Donaldson, M. Dijak, E. Bevis, R. Pandeya, W. Keller, S. Gleddie. 1991. Fertile somatic hybrids between transgenic *N. tabacum* and transgenic *N. debneyi* selected by dual-antibiotic resistance. Theor. Appl. Genet. 82:450-6.

Trick, M., Heizmann, P. 1992. Int. Rev. Cytol. 140, 485-524.

Uchimaya H, S. Kidou, T. Shimazaki, S. Aotsuka, S. Takamatsu, R. Nishi, H. Hashimoto, Y. Matsubayashi, N. Kidou, M. Umeda, A. Kato. 1992. Random sequencing of cDNA libraries reveals a variety of expressed genes in cultured cells of rice (*Oryza sativa* L.). Plant J. 2:1005-1009.

Van Deynze, A.E., B. Landry and K.P. Pauls. 1994. The identification of restriction fragment length polymorphisms linked to seed coat colour genes in *Brassica napus*. Genome 38:534-542.

Yasuda, E., Ebinuma, H., Wabiko, H. 1997. A novel glycine-rich/hydrophobic 16 kDa polypeptide gene from tobacco: similarity to proline-rich protein genes and its wound-inducible and developmentally regulated expression. Plant Mol Biol. 33, 667-678.

REACH AND IMPACT

At the completion of the project, we have evaluated the effectiveness of various molecular approaches to increasing *Fusarium* resistance in corn. We have established proof of concept that the action of DON, which is a major factor in pathogenesis by *Fusarium graminearum*, could be reduced by the modification of a specific protein (RPL3) involved in protein synthesis in the corn cell. This was clearly established in tobacco and corn cells in tissue culture. When the modified gene was expressed in these cells a tolerance to DON was observed. Since DON has been previously demonstrated to be a virulence factor for Fusarium when it attacks corn and wheat under field conditions we can hypothesize that this modified gene will confer some tolerance to corn under field conditions. Evaluation of corn lines transformed with *Rpl3:c258* will continue in field trials this summer. A US patent (#6,060,646) has been issued for this technology, an additional continuation-in-proof has been filed, and office action for examination in other applicable countries are pending. This finding opens the door to several strategies to manage the effect of DON, by either direct modification of one copy of the *Rpl3* gene family or by insertion of a modified gene. One complication with this strategy to genetically modify disease resistance is the complexity of the eukaryotic ribosome complex which would be operative in corn plants. The demonstration of disease attentuation by this modified gene remains to be proven, but this is a very promising avenue to elevating the level of host plant resistance. This research has led to many requests for material transfers of this gene for maize and other monocot transformation.

To optimize the expression of this gene, we have also isolated a silk-specific promoter which could be used to specifically target the expression of the modified gene to tissue susceptible to *Fusarium* attack. A patent for this promoter has been filed in the US. The isolation and characterization of other tissue-specific promoters (maize ear) is underway. In addition, in the study of maize-*Fusarium* interactions, the genetic response of the plant to invasion by the fungi was studied and novel genes expressed will be characterized further and their role as candidate genes for marker development will be assessed. The

possibility of building up resistance by constitutive over-expression of these genes to provide an added level of resistance will be explored. The refinement of inoculation and tissue collection protocols for this study has formed the basis for our maize/*Fusarium* genomics project which utilizes SSH (suppressive subtractive hybridization) to enrich cDNA libraries for genes expressed upon fungal inoculation, accumulating a maize EST database, microarray analysis, and other tools in gene discovery. The genes isolated using differential display will be useful markers of expression in future microarray studies.

This funding has permitted the establishment of molecular methodologies to study in detail the complex interaction between this very serious Canadian pathogen (*Fusarium graminearum*) and corn under Ontario field conditions. We have well established corn gene transfer methods which have allowed the testing and evaluation of two candidate genes in our maize inbred lines.

This funding has allowed us to train several young Canadians in the field of molecular pathology and plant-fungal interactions. We have presented the results of this work at several international and national symposia, workshops, and annual meetings of scientific societies. The patents issued and filed in Canada, and worldwide will allow these technologies to be used by this team, and other Canadians for the improvement of maize and possibly other cereals. We have also made several presentations to growers, producers, and other industry researchers.

TECHNOLOGY TRANSFER AND COMMUNICATIONS

Patents

Harris, L.J., Gleddie, S.C. Tolerance of trichothecene mycotoxins in plants and animals throught the modification of the peptidyl transferase gene. U.S. Patent #6,060,646 issued May 9, 2000

Harris, L.J., Gleddie, S.C. 2000. Tolerance of trichothecene mycotoxins in plants through the modification of the ribosomal protein L3 gene. US patent filed in continuation and pending.

Ouellet, T., Singh, J. and Tao, T. 2000. Corn silk gene and regulatory region. US patent filed in continuation and pending.

Material Transfers

Modified rice *Rpl3* gene for maize transformation; and fi clones for RFLP mapping work (P. Pauls, U. Guelph)

Modified rice *Rpl3* gene for wheat transformation (M. Jordan, CRC, Winnipeg AAFC)

Modified rice *Rpl3* gene for barley and durum wheat transformation (R. Chibbar, PBI/NRC, Saskatoon)

Modified rice *Rpl3* gene for monocot transformation, excluding corn (W. G. Thompson's)

Modified rice *Rpl3* gene for maize transformation (Currently under negotiation with Pioneer Hi-Bred)

Invited talks

Harris, L.J., Gleddie, S.C., Simmonds, J., Ouellet, T., and Singh, J. 1998. *Fusarium* resistance via biotechnology. Novartis Corporation, Raleigh, NC.

Harris, L.J. 1999. Molecular studies of the *Z. mays*/ *F. graminearum* interaction. Canadian Phytopathological Society Meeting "Eastern Cereal Pathology in the 21st Century", Ottawa, ON.

Harris, L.J., Gleddie, S.C., Simmonds, J., Ouellet, T., and Singh, J. 1999. *Fusarium* resistance via biotechnology. Pioneer Hi-Bred, Johnston, IA.

Gleddie, S.C. and Harris, L.J. 1999. Recent progress in molecular strategies to improve the *Fusarium* tolerance of plants. Canadian Fusarium Head Blight Workshop, Winnipeg, MAN.

Harris, L.J. 2000. 1) Possible molecular strategies for engineering resistance to fusarium head blight AND 2) *Fusarium* resistance via biotechnology overview. Eastern Expert Committee on Cereals and Oilseeds, Ottawa, ON.

Harris, L.J., and Gleddie, S.C. 2000. Developing plant tolerance to trichothecene mycotoxins using a transgenic approach. Phytopathology 90:s96. American Phytopathological Society Annual Meeting, August, 2000, New Orleans, LA.

Harris, L.J., and Gleddie, S.C. 2001. Ribosomal protein gene modification as a potential *Fusarium* resistance strategy. 5th Canadian Plant Tissue Culture & Genetic Engineering Workshop, May, 2001, Saskatoon, SK.

Harris, L.J., and Gleddie, S.C. 2001. Ribosomal protein transgene to increase plant tolerance to trichothecenes. Gordon Research Conference "Mycotoxins & Phycotoxins", June, 2001, Williamstown, MA.

Publications

Harris, L.J. 1999. *Fusarium* Resistance Via Biotechnology. Ontario Corn Producer, July issue.

Harris,L.J., Gleddie, S.C. 2001. A modifed *Rpl3* gene from rice confers tolerance of the *Fusarium graminearum* mycotoxin deoxynivalenol to transgenic tobacco. Physiol. Mol. Plant Pathol. (In press).

Poster presentations

Harris, L.J., Gleddie, S.C., and Simmonds, J.A. 1998. Increased tolerance of *Fusarium* trichothecene mycotoxins in transgenic plants. Cambridge Healthtech "Impact of Molecular Biology on Crop Production", Minneapolis, MN.

Harris, L.J., Saparno, A., Allard, S., Koul, A., and Ouellet, T. 1999. Genes induced during *Fusarium graminearum* infection of maize. 41st Maize Genetics Conference, Lake Geneva, WI.

Ouellet, T., Saparno, A., Koul, A., Allard, S., and Harris, L.J. 1999. Genes induced in *F. graminearum*/*Zea mays* interactions. 20th Fungal Genetics Conference, Asilomar, CA.

Ouellet, T., Saparno, A., Koul, A., Zhu, L., Allard, A., and Harris, L.J. 1999. Genes induced during early infection in pink ear rot of maize. American Phytopathological Society Meeting, Montreal.

Ouellet, T., Saparno, A., Koul, A., Zhu, L., Allard, S., and Harris, L.J. 1999. Genes induced during early infection of maize ears by *Fusarium graminearum*. Canadian Fusarium Head Blight Workshop, Winnipeg, MAN.

Ouellet, T., Saparno, A., Koul, A., Zhu, L., Allard, S., and Harris, L.J. 1999. Genes induced during early infection in pink ear rot of maize. Ottawa Life Sciences National Conference, Ottawa, ON.

Ouellet, T., Koul, A., Allard, A., Saparno, A., Dadej, K. and Harris, L. 2000. Cloning and characterization of genes involved in Gibberella ear rot of maize. Keystone Symposia on Signals and Signal perception in biotic interactions in plants, Taos, NM.

Harris, L.J., Allard, A., Saparno, A., Koul, A., and Ouellet, T. 2000. Maize/*Giberella* ear rot - maize genes induced in the plant/pathogen interaction. 42st Maize Genetic Conference. Couer d'Alene, ID.

Harris, L.J., Gleddie, S.C. 2000. A modified *Rpl3* gene from rice confers tolerance of the *Fusarium graminearum* mycotoxin deoxynivalenol to transgenic tobacco. 6th International Congress of Plant Molecular Biology, Quebec City, QC

Ouellet, T., Koul, A., Allard, A., Saparno, A., Dadej, K. and Harris, L.J. 2000. Maize genes induced in maize/Gibberella ear rot interaction. 6th International Congress of Plant Molecular Biology, Quebec City, QC.

Tao, T., Ouellet, T. and Singh, J. 2000. Isolation of a novel glycine rich protein (GRP) from *Zea mays* silk tissue. 6th International Congress of Plant Molecular Biology, Quebec.

PARTNERSHIPS AND PERSONNEL

The current work was planned and executed as a partnership between Agriculture and Agri-Food Canada, Ottawa and The Plant Agriculture Dept at the University of Guelph. The work in Ottawa was carried out in the labs, fields, and greenhouses of Building #20 and #21 of the Eastern Cereal & Oilseed Research Centre, Central Experimental Farm. The five scientists directly responsible for deliverables were Drs. Steve Gleddie, Linda Harris, Thérèse Ouellet, John Simmonds, and Jas Singh. We would like to acknowledge the technical advice and support of Drs. Lana Reid, Marc Savard, and Shea Miller. The *Rpl3:c258* gene, C3 silk-specific promoter and fi genes were isolated and characterized in Ottawa and are the property of AAFC. The funding for this research is derived from a corn consortium which was formed under the auspices of OAFT and includes funding from Canadian Adaption Council (CanAdapt program), Ontario Research Enhancement Program and AAFC MII. Private funding comes from members of the corn consortium including the OCPA, Ontario Pork Producers, Novartis, Pioneer Hi-Bred, King Agro and other members of the consortium.

Table 7. Personnel involved directly in this project. There have also been 9 university students working for 16 week periods on this project (Swann, Yip, Yajima, Ferrer, Lam, Harrison, Parker, and Jim ?).

Name	Position/Level	Project*	Funding/Percentage of time	Time of employment
S. Allard	Technician/EG-05	RPL3,PFI	A-base/40%	continuous
R. Barrette	Technician/EG-04	RPL3	OCPA-MII/100%	11/97-11/99
L. Cass	Technician/EG-05	OXO, MT, RPL3	A-base/40%	continuous
K. Dadej	Technician/EG-04	PFI	OCPA-MII/100%	07/99 - current
C. Gagnon	Technician/EG-04	RPL3,ScFv	OCPA-MII/100%	08/00-current
A. Hermans	Technician/EG-05	Rpl3, ScFv	A-base/40%	continuous
I. Joanisse	Technician/EG-03	RPL3, MT	OCPA-MII/100%	08/99 - current
A. Johnston	Technician/EG-04	PFI	OCPA-MII/100%	07/00 - current
A. Kathiresan	PDF	PFI	OCPA-MII/100%	12/97-09/98
S. Kelso	Technician/EG-03	RPL3, MT	OCPA-MII/100%	07/98-07/99
A. Koul	Technician/EG-05	PFI	A-base/40%	continuous
J. Lachance	Technician/EG-03	RPL3, MT	OCPA-MII/100%	03/98-06/00
A. Saparno	Technician/EG-04	PFI	OCPA-MII/100%	12/97-01/00
J. Skene	Technician/EG-04	ScFv	OCPA-MII/100%	11/97-06/00
T. Tao	Technician/EG-04	Prom	OCPA-MII/100%	10/97-current
L. Zhu	Biologist/BIO-01	PFI	OCPA-MII/100%	01/99-06/99

*Project: Rpl3 = Toxin-based resistance strategy: Modified Rpl3 gene
ScFv = Toxin-based resistance strategy: DON-specific ScFv gene
OXO = Broad Spectrum Disease Resistance: OXO gene
MT = Maize transformation
Prom = Promoter isolation and testing
PFI = Plant/Fusarium interactions

Fusarium Resistance and Genetic Improvement in Ontario Corn through Biotechnology

Statement of Project Expenditures: April 1, 2000 through March 31, 2001

Project Activity Area / Item	Phase II Base Project Cash	In-Kind	*SubTotal*	Overal **Total**	Notes:
a. Transformation of Ontario corn for increased resistance to					
1. Neutralization of factors specific to Fusarium					Skene and Gagnon
Research technicians (2)salary+student	66				
Consumables for DNA and protein	30				
Sub-Total	***96***	***0***	-	***0***	
2. Increasing overall resistance to Fusarium (Ottawa)					
DNA analysis consumables	10				
Growth Cabinet greenhouse		20			
Sub-Total	***10***	***20***	-	***0***	
3. Plant Transformation Technologies					Lachance and Joanisse
i) Particle gun (Ottawa)					and
Research technician (2) salary and Student (1)	124				students (Harrison &)
DNA analysis and tissue culture consumables					
Mycotoxin analyses(0.25 technical p.y.)		23			
Mycotoxin analyses(0.25 technical p.y.)					
Field testing					
Growth cabinet/tissue culture room/ greenhouse costs		50			
Sub-Total	***147***	***50***	-	***0***	
ii) Agrobacterium transformation (Guelph)					
Post-doctoral fellow (1) Salary and					
Molecular vector construction					
Technician salary (1) and benefits					
Microspore culture (technician salary)					
Tissue culture consumables					
Growth cabinet and Growth room costs					
Field Testing					
Sub-Total	***0***	***0***	-	***0***	
4. Enabling Technology: Promoter analysis (Ottawa)					
Research Technician (1) Salary and	17				Tao
DNA analysis consumables	15				
Growth cabinet and greenhouse costs		20			
Sub-Total	***32***	***20***	-	***0***	

5. Plant/Fusarium Interactions (Ottawa)					
Research technicians (2) salary and	49				Dadej, Johnston
DNA analysis consumables	15				and students (Lam and Parker)
Growth cabinet and greenhouse		22.5			
Sub-Total	***64***	***22.5***	-	***0***	
b. Identifying Molecular markers for Improving Ontario (northern) corn					
Post-doctoral fellow (1) salary and					
Technician salary (1) and benefits					
consumables DNA analysis					
greenhouse/Growth room					
Field costs 4)					
Equipment; repair & replacement					
Travel/Shipping					
Sub-Total	***0***	***0***	-	***0***	
Overhead(10%)	35				
Networking	5				
Overall Project Costs: 2000/2001	***389***	***112.5***	-	***0***	